

**Recognition of Environmental Conditions Influences**  
***Francisella*-Macrophage Interactions**

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Paul Edward Carlson Jr., PhD

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*Francisella tularensis*, the causative agent of tularemia, is a pathogen capable of survival and growth in a vast array of environments ranging from arthropod vectors to over one hundred different mammalian hosts, including humans. An understanding of the mechanisms that this bacterium uses to adapt to these varied environments is vital to fully understanding its pathogenesis. The environmental signals that *Francisella* responds to include low iron concentrations, oxidative stress, and temperature. The work described in this dissertation constitutes a significant step forward in our understanding of *Francisella* adaptation specifically to the host intracellular environment. We have shown that bacterial growth conditions have a great impact on bacterial phenotypes, particularly on the ability of *Francisella* to induce or inhibit macrophage cytokine production. We have identified a specific eukaryotic molecule, spermine, which is abundant in the intracellular environment and leads to significant changes in bacterial phenotypes and gene expression. We also present the first evidence of a role for the abundant *Francisella* IS elements in the regulation of transcription by functioning as spermine-responsive promoters. The ability of the bacterium to adapt to this signal is vital to its survival and a mutant lacking this sensing mechanism is highly attenuated. A thorough analysis of different culture conditions has led to the identification of both known and putative virulence factors that may be important for altering host cell responses to *F. tularensis*. Our results

demonstrate a novel mechanism of host-pathogen interaction and could have significant implications for other intracellular pathogens.

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	XII
1.0 INTRODUCTION.....	1
1.1 THE <i>FRANCISELLAE</i> .....	2
1.1.1 Genetic diversity among <i>Francisella</i> subspecies .....	4
1.2 <i>FRANCISELLA</i> AS A PATHOGEN.....	6
1.2.1 Symptoms of Tularemia .....	6
1.2.2 Environmental life cycle and transmission of <i>Francisella</i> .....	8
1.2.3 The intracellular lifestyle of <i>Francisella</i> .....	11
1.2.4 Virulence determinants of <i>Francisella</i> .....	15
1.3 IMMUNE RESPONSE TO <i>FRANCISELLA</i> .....	19
1.3.1 Mechanisms of host cell recognition of a <i>Francisella</i> infection.....	19
1.3.2 Role of Cytokines and Chemokines.....	22
1.3.3 Role of B cells and specific antibodies in <i>Francisella</i> immunity.....	23
1.3.4 Role of T cell mediated immunity in <i>Francisella</i> infection .....	24
1.3.5 Immune modulation by <i>Francisella</i> .....	26
1.4 <i>FRANCISELLA</i> RESPONSE TO ENVIRONMENTAL SIGNALS.....	27
1.5 POLYAMINES .....	29
1.5.1 Polyamine synthesis .....	30

1.5.2	Transport of polyamines .....	31
1.5.3	Function of polyamines in bacterial systems .....	33
1.5.4	Role of polyamines in bacterial virulence.....	35
1.6	STATEMENT OF THE PROBLEM ADDRESSED IN THIS THESIS .....	36
2.0	CHAPTER 1: MODULATION OF VIRULENCE FACTORS IN <i>FRANCISELLA TULARENSIS</i> DETERMINES HUMAN MACROPHAGE RESPONSES. ....	38
2.1	ABSTRACT: .....	38
2.2	INTRODUCTION: .....	40
2.3	MATERIALS AND METHODS:.....	42
2.4	RESULTS:.....	48
2.4.1	Isolation of a novel LVS variant. ....	48
2.4.2	Human macrophage response to <i>Francisella</i> isolates. ....	48
2.4.3	Molecular characterization of LVS isolates. ....	53
2.4.4	Reversion of ACV to the LVS phenotype. ....	60
2.5	DISCUSSION:.....	63
3.0	CHAPTER 2: DETECTION OF THE HOST CYTOSOLIC ENVIRONMENT IS ESSENTIAL FOR <i>FRANCISELLA</i> SURVIVAL .....	68
3.1	ABSTRACT: .....	68
3.2	INTRODUCTION: .....	70
3.3	MATERIALS AND METHODS:.....	72
3.4	RESULTS:.....	80
3.4.1	Human macrophage response to <i>Francisella</i> following cultivation in the presence of spermine: .....	80

3.4.2	<i>Francisella</i> gene expression changes in response to spermine:.....	82
3.4.3	Role of <i>Francisella</i> IS elements in the response to spermine:.....	88
3.4.4	Spermine responsive genes are regulated during intramacrophage growth:	90
3.4.5	Disruption of the <i>potF</i> gene eliminates <i>Francisella</i> response to spermine:	93
3.5	DISCUSSION:.....	100
4.0	CHAPTER 3: GLOBAL EFFECT OF CULTURE CONDITIONS ON <i>FRANCISELLA TULARENSIS</i> GENE EXPRESSION. ....	106
4.1	ABSTRACT.....	106
4.2	INTRODUCTION .....	108
4.3	MATERIALS AND METHODS .....	110
4.4	RESULTS AND DISCUSSION .....	114
4.4.1	Effect of growth conditions on <i>F. tularensis</i> LVS:.....	114
4.4.2	Global gene expression changes induced in different growth media:..	117
4.4.3	Altered expression of <i>Francisella</i> pathogenicity island genes: .....	126
4.5	CONCLUSIONS .....	129
5.0	SUMMARY .....	130
	BIBLIOGRAPHY .....	136

## LIST OF TABLES

Table 1: Differences in <i>Francisella</i> species and subspecies.....	3
Table 2: Macrophage viability following incubation with LVS isolates.....	53
Table 3: MALDI-TOF identification of proteins present in LVS but reduced in ACV .....	58
Table 4: Plasmids, strains, and primers used in this chapter. ....	79
Table 5: <i>F. tularensis</i> genes exhibiting increased expression in response to spermine <sup>a</sup> .....	86
Table 6: <i>F. tularensis</i> genes exhibiting decreased expression in response to spermine <sup>a</sup> .....	87
Table 7: Genes uniquely up-regulated in each media type.....	119
Table 8: Genes uniquely down-regulated in each media type.....	120

## LIST OF FIGURES

Figure 1: Environmental life cycle and transmission of <i>Francisella</i> .....	9
Figure 2: Intracellular life cycle of <i>Francisella</i> .....	12
Figure 3: Schematic representation of the genomic organization of the <i>Francisella</i> pathogenicity island.....	17
Figure 4: Chemical structure of common polyamines.....	29
Figure 5: Bacterial polyamine biosynthetic pathway.....	31
Figure 6: Polyamine transport in bacteria.....	33
Figure 7: Proinflammatory cytokine production by human monocyte derived macrophages following exposure to <i>F. tularensis</i> LVS and ACV.....	49
Figure 8: Macrophage response to live bacteria and bacterial lysates.....	50
Figure 9: Inhibition of macrophage TLR signaling by LVS.....	52
Figure 10: Immunoreactivity of LPS from LVS and ACV.....	54
Figure 11: Differences in protein expression between LVS and ACV.....	55
Figure 12: Identification of differences in the soluble protein fractions of LVS and ACV by two-dimensional gel electrophoresis.....	57
Figure 13: Quantification of Ig1C levels by immunoblot.....	59
Figure 14: ACV reversion to the LVS phenotype.....	61

Figure 15: Inhibition of macrophage cytokine response by <i>Francisella</i> grown in the presence of spermine.....	81
Figure 16: <i>Francisella</i> gene expression changes induced by spermine.....	84
Figure 17: <i>Francisella</i> IS elements contain spermine-responsive promoters.....	89
Figure 18: Regulation of spermine responsive gene during intracellular growth.....	92
Figure 19: Mutation of <i>potF</i> gene in <i>F. tularensis</i> LVS.....	94
Figure 20: Attenuation of the <i>F. tularensis</i> LVS <i>potF</i> mutant. ....	98
Figure 21: Model of <i>Francisella</i> response to spermine in macrophages.....	105
Figure 22: Culture media has dramatic effects on <i>Francisella</i> phenotypes.....	116
Figure 23: Unique <i>Francisella</i> gene expression changes induced by culture conditions.....	118
Figure 24: Categorical analysis of uniquely induced genes.....	123
Figure 25: Influence of media type on expression of genes within the <i>Francisella</i> pathogenicity island.....	127

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## 1.0 INTRODUCTION

*Francisella tularensis*, the causative agent of tularemia, is a facultative intracellular gram negative bacterium [1]. The high virulence of this bacterium has led to its classification as a Category A biodefense agent by the Centers for Disease Control. In fact, *F. tularensis* based biological weapons were developed by Japan, the former Soviet Union, and the United States between 1930 and 1960 [2]. Although much research has been performed examining *Francisella*, the current knowledge of its pathogenesis remains incomplete. As a successful pathogen, *Francisella* can be found in a wide range of hosts, arthropod vectors, and environments [3]. *F. tularensis* is also capable of survival and growth in a variety of cells, including those designed to kill microorganisms, such as macrophages and dendritic cells [4-8]. Recent research has identified a *F. tularensis* pathogenicity island that is required for intracellular growth [9] and has started to elucidate the intricacies of gene regulation in this pathogen [10-13]. A pathogen with such a diverse range of potential environments must have mechanisms to identify its surroundings. This thesis describes our laboratory's work in elucidating the mechanisms *Francisella* uses to respond to its environment, which led to the identification of a eukaryotic signal, spermine, that induces bacterial adaptation to the host intracellular environment.

## 1.1 THE *FRANCISELLAE*

There are currently four known subspecies of *Francisella tularensis*; *tularensis*, *holarctica*, *mediasiatica*, and *novicida*, which cause the disease Tularemia. Each of these members differs from the others in a variety of ways, including virulence, global localization, and transmission (Table 1) [14]. There are significant differences in their global localization and the severity of disease symptoms. Type A strains, including *F. tularensis* subsp. *tularensis* are the most virulent members of this species and cause a more severe form of tularemia. Infection with this subspecies leads to severe disease in human and other mammals, and is often fatal without antibiotic therapy. *F. tularensis* subsp. *tularensis*, which is predominantly found in North America, has recently been split into two classes; Type AI (A-east) and Type AII (A-west), based on observed differences in disease severity and localization within the United States [15]. Type B strains including *F. tularensis* subsp. *holarctica* are significantly less virulent. Though it does lead to severe symptoms, the disease is less likely to be fatal in humans. This subsp. is more widespread than subsp. *tularensis* and is found throughout North America and Eurasia [3,16,17]. The third member of the *Francisellae* is *F. tularensis* subsp. *mediasiatica*, a relatively limited bacterium that has only been isolated in Kazakhstan and Turkmenistan [3]. This subspecies exhibits virulence similar to subsp. *holarctica* in rabbit models of infection, though human data are unavailable [18].

**Table 1: Differences in *Francisella* species and subspecies.**

<i>Francisella</i> species	Virulence in mice	Virulence in humans	Global distribution
<i>F. tularensis</i> :			
subsp. <i>tularensis</i> (Type A)	+++++	+++++	North America
subsp. <i>holarctica</i> (Type B)	++++	+++	Northern Hemisphere
subsp. <i>mediasiatica</i>	++++	ND <sup>b</sup>	Central Asia
<i>F. novicida</i>	+++	+	Rare - North America
<i>F. philomiragia</i>	NT <sup>a</sup>	+	Rare - North America

<sup>a</sup> – NT= not tested

<sup>b</sup> – ND= no data available

Another member of the *Francisella* genus is *F. novicida*. This bacterium is officially a separate species within the genus, although it has been suggested that it be reclassified as a subspecies of *F. tularensis* based on genomic sequencing results [3]. *F. novicida* is found primarily in North America, though it has also been isolated in Australia [19]. Although some cases have been reported, it is rare that this subspecies causes disease in humans [19,20].

The only other current member of the *Francisella* genus is *F. philomiragia*. An organism with minimal virulence in humans, it has not been studied extensively. Cases of *F. philomiragia* have been reported in North America and Europe, but this bacterium has been primarily associated with disease in immunocompromised individuals [20-26]. Though these are the only members of this family that have been identified to date, recent research has been identifying *Francisella*-like organisms from a variety of sources [27-31], which will likely lead to the inclusion of more organisms in the *Francisella* genus in the near future.

### 1.1.1 Genetic diversity among *Francisella* subspecies

The *F. tularensis* subspecies exhibit extensive variation at the genome level, including point mutations, gene silencing, and genomic rearrangements, which influence the phenotypic differences observed among these closely related organisms [32]. Genomic rearrangement is surprisingly frequent comparing *F. tularensis* subsp. *tularensis* with *F. tularensis* subsp. *holarctica*, and likely the result of recombination between IS elements within the genome [17]. Over 100 copies of these mobile genetic elements, which encode proteins with predicted transposase activity, are represented in each *Francisella* subspecies [32,33]. The frequency of IS elements in the *Francisella* genomes results in an increased potential for such recombination events [32,33]. Despite this recombination potential, a high degree of sequence similarity remains between subspecies [3].

Numerous pseudogenes have been identified within the *Francisella* genomes [3,17,32,33]. This loss of genetic information is thought to be a mechanism of evolution by these bacteria, eliminating all genes that are unnecessary for the intracellular environment [3,17,32,33]. *F. tularensis* subsp. *holarctica* appears to have experienced more rapid or extensive adaptation through this mechanism because the genomes of this subspecies contain about 300 pseudogenes, approximately 100 more than have been identified in *F. tularensis* subsp. *tularensis* [17].

Multiple experimental methods have been used to determine the evolutionary path of *Francisella*. Comparative genomic hybridization has identified eight “regions of difference” between *Francisella* subspecies, which have been lost throughout the evolution of this organism [16]. While *F. tularensis* subsp. *tularensis* genomes contain all of these regions, the DNA content of *F. tularensis* subsp. *holarctica* strains exhibited loss of many of these regions [16].

Another study compared the genomes of pathogenic *Francisella* (subsp. *tularensis* and *holarctica*) with non-pathogenic *F. novicida*. This comparison identified 46 genes that are unique to the pathogenic strains and 28 genes that are present in only the more virulent strain, *F. tularensis* subsp. *tularensis* [32]. This analysis also identified 160 genes in *F. novicida* that are non-functional in the more virulent strains, supporting the idea of evolution by genetic decay [32]. Genomic analyses of *F. philomiragia* have not yet been performed.

## 1.2 *FRANCISELLA* AS A PATHOGEN

*F. tularensis* infection leads to a potentially severe disease, tularemia. These bacteria are found throughout the majority of the northern hemisphere, however the incidence of human disease varies greatly throughout this area [3]. Although the total number of reported cases of tularemia in the United States is low, there are “hotspots” of infection around the country where cases are more common [34-36]. The island of Martha’s Vineyard, Massachusetts is one such location, where a relative high incidence of *Francisella* infection has been reported, particularly in people with exposure to animals that may have been infected [34,35,37]. Between 2000 and 2005, 778 cases of human tularemia were reported in the United States. These numbers include cases of both Type A and Type B tularemia. Cases of tularemia are more frequently reported throughout Europe, especially in eastern European countries. Sweden and Finland reported 1,828 and 2,697 cases of *F. tularensis* subsp. *holarctica* infection between 2000 and 2005 [18,38,39]. Outbreaks of tularemia have also been reported in Turkey [40-42] and Russia [18]. A high incidence of cases was reported in Russia during World War II, an outbreak rumored to be associated with an intentional release of *F. tularensis* [43].

### 1.2.1 Symptoms of Tularemia

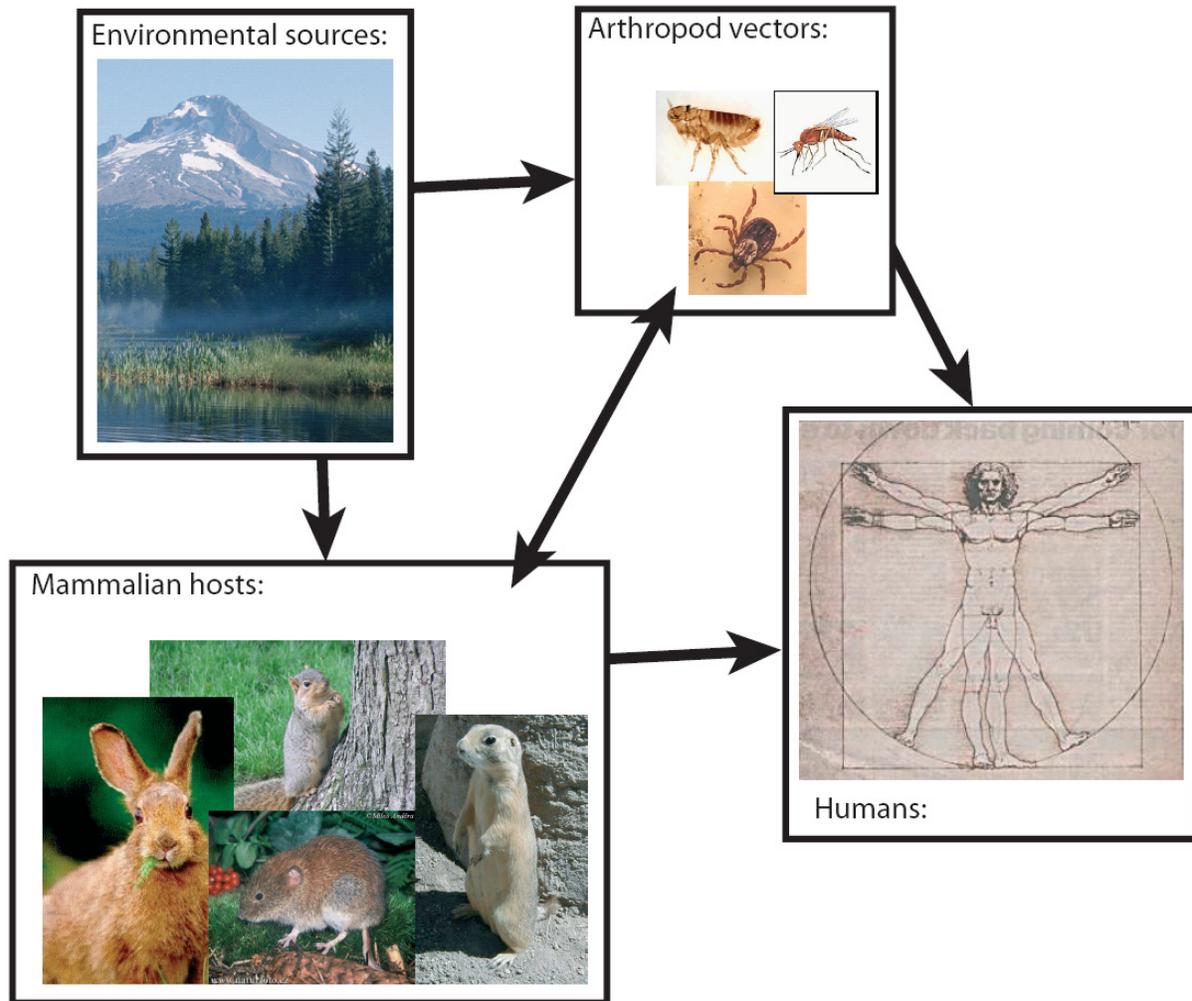
The severity of symptoms depends on both the infecting strain and the route of infection. *Francisella* can infect a host through a range of routes, including cutaneous, mucosal,

gastrointestinal, and pulmonary routes of inoculation [1]. Symptoms of tularemia usually begin 3-5 days following infection with *Francisella*. Infection by any route leads to general symptoms including fever, malaise, chills, and headache [2]. Infection through cutaneous or mucosal routes, which is often the result of vector-borne transmission, leads to the development of ulceroglandular tularemia, the predominant form of tularemia seen in Europe. This type of tularemia is characterized by the presence of a primary ulcer at the initial site of infection. The draining lymph nodes will then become enlarged and tender. If antibiotics are not administered within approximately one week, severe lymph node swelling may lead to abscess formation in 30-40% of cases [18]. Oculoglandular tularemia is caused by infection of the conjunctiva, brought about by direct contact of bacteria with the eye. Though these infections are very rare, they lead to symptoms similar to ulceroglandular tularemia including ulcers and nodules on the conjunctiva and regional lymph node swelling [1]. Ingestion of contaminated food or water can lead to both oropharyngeal and gastrointestinal tularemia. Oropharyngeal tularemia typically presents as a severe sore throat and is often accompanied by regional lymph node swelling [1]. Gastrointestinal infection can range from a mild diarrhea to an acute, possibly fatal disease depending on infectious dose [1]. Pneumonic tularemia, the most severe form of *Francisella* infection, can be acquired through the inhalation of contaminated aerosols or through secondary spread through the body from the initial site of inoculation [2]. *Francisella* can be easily aerosolized, as has been reported in a recent outbreak associated with landscaping on Martha's Vineyard [34,35]. Inhalation of as few as ten bacteria can lead to pneumonia that can be difficult to diagnose with conventional chest radiographs, particularly early after infection [2]. The symptoms of respiratory tularemia vary depending on infecting strain. These usually consist of a systemic infection with very high fever and often do not include any signs of respiratory disease

[14]. Infection with *F. tularensis* subsp. *holarctica* results in a mild, non-fatal form of respiratory disease. In contrast, infection with the more virulent *F. tularensis* subsp. *tularensis* leads to severe disease characterized by high fever, chills, malaise and cough [14]. Diagnosis of tularemia early in infection is vital, especially in the case of pneumonic tularemia, which has been associated with mortality rates up to 60% if left untreated [18].

### **1.2.2 Environmental life cycle and transmission of *Francisella***

Elucidation of the environmental sources of *Francisella* and its transmission cycle is a complex process. One of the most interesting aspects of *Francisella* biology is its association with a wide range of environments, including a plethora of arthropod and mammalian hosts. Members of the *Francisellae* have been associated with 190 species of mammals and 23 bird species [3]. *Francisella*-related bacteria have also been isolated from fish, reptile, and amphibian species [3,27,44,45]. Over 88 different invertebrate species have been implicated in transmission of *Francisella*, including biting flies, fleas, ticks, and mosquitoes [3]. The broad host and vector range of this pathogen makes it difficult to identify environmental niches or a certain species that acts as a natural reservoir of *Francisella*, further exacerbating the task of determining the natural transmission cycles of this microorganism (summarized in Figure 1).



**Figure 1: Environmental life cycle and transmission of *Francisella*.**

*Francisella* can be isolated from aquatic environments, various arthropod species, and many different species of mammal. Bacteria can be transmitted from mammals to other mammals or humans either through direct contact, inhalation of aerosols, or the bite of an infected arthropod.

Hosts and vectors associated with *Francisella* in North America:

The presence of both *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* throughout North America has caused difficulty in identifying hosts specific to each subspecies [3]. Although *Francisella* can infect many mammal and bird species, the primary hosts of this

bacterium in North America are thought to be lagomorphs, including rabbits, hares, and jackrabbits [3]. *F. tularensis* subsp. *holarctica* has also been associated with voles, American beavers, and muskrats on this continent [3].

More information is available regarding the arthropod vectors that transmit *Francisella* in North America. In the United States, there is a geographic difference in the arthropod species primarily responsible for *Francisella* transmission. The arthropods most important to *Francisella* transmission, both between mammals and from mammal to humans, include three tick species and one fly species. In the western United States, one tick species *Dermacentor andersoni* and the deer fly, *Chrysops discalis* are the primary sources of arthropod borne tularemia. Throughout the rest of the country, the ticks *Amblyomma americanum* and *D. variabilis* are chiefly responsible for transmission of *Francisella* [3].

#### Hosts and vectors associated with Type B strains of *Francisella* in Eurasia:

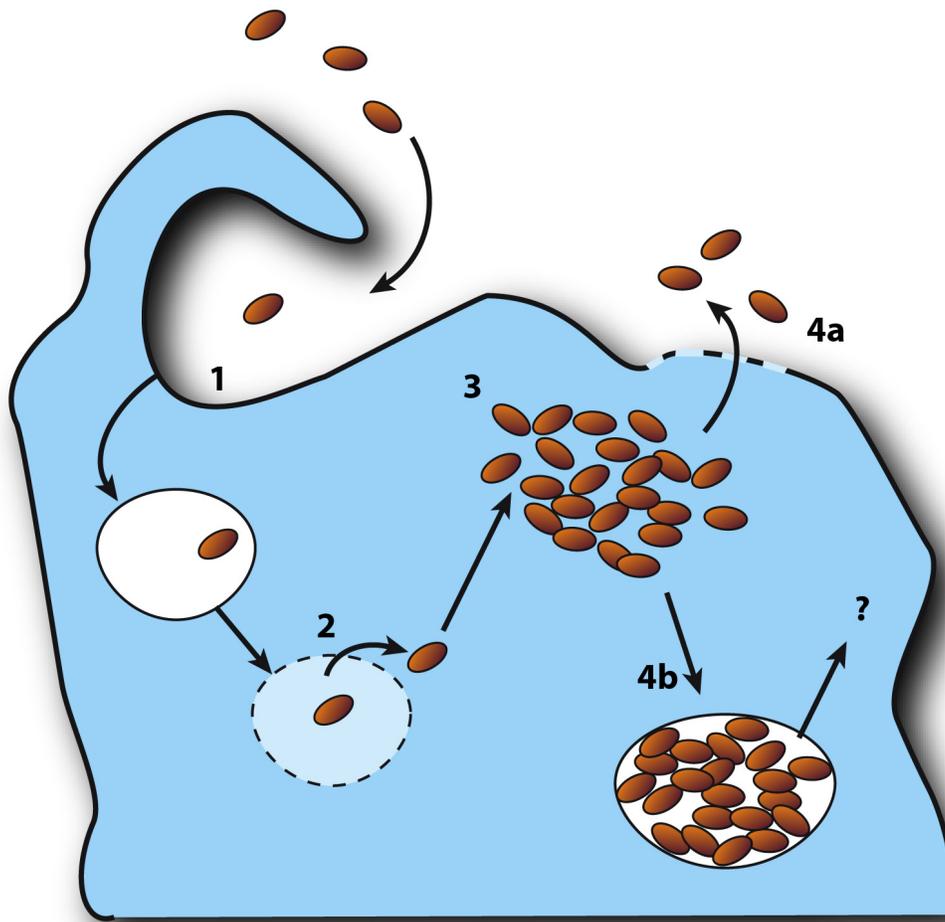
Much like North America, many small mammals have been associated with *Francisella* infections in Europe and Asia. The species most commonly infected with *Francisella* are hares and rodents. Many reported cases of tularemia have been associated with hunting and skinning of these mammals [3,46]. The brown hare (*Lepus europaeus*) is considered the major source of tularemia in central Europe while the mountain hare (*Lepus timidus*) is the most common source in colder European climates [47]. The other major carriers of tularemia in Eurasia are voles, with *Francisella* being carried by multiple species [3].

Transmission of type B strains of *Francisella* is thought to be highly dependent on blood-feeding arthropods including ticks, mites, tabanid flies, mosquitoes, and fleas [3]. Multiple species of tick are believed to be important for the maintenance of *Francisella* in mammal

populations throughout Eurasia, with *D. reticulatus* being the most common carrier [48,49]. Unlike the North American ticks, these arthropods are thought to play only a minor role in the transmission of *Francisella* to humans with only one percent of total cases being the result of a tick bite [49]. Human tularemia outbreaks in northern Europe are more commonly associated with mosquito bites [50]. Ten species of mosquitoes from the genera *Aedes*, *Culex*, and *Anopheles* have been found carrying *Francisella* [3]. Experimental studies have shown that these species are able to carry and transmit the bacteria for long periods of time (up to 35 days), however, no evidence of *Francisella* replication within these vectors has been reported [3].

### **1.2.3 The intracellular lifestyle of *Francisella***

Once *Francisella* enters a host, it will be taken up by phagocytes near the site of infection. These cells then become the primary site of bacterial replication. Although macrophages are commonly thought to be the primary cell type infected by *Francisella*, it is becoming clear that this bacterium is capable of replication in a variety of cell types, including dendritic cells, fibroblasts, endothelial cells, hepatocytes, and muscle cells [51]. Knowledge of a pathogen's movement throughout the host during infection is vital to understanding the pathogenesis of the organism. Studies examining intracellular progression of *Francisella* in macrophages have shown that the bacterium undergoes 4 primary steps in its intracellular lifecycle [51] including; 1.) Entry, 2.) Phagosomal escape, 3.) Replication in the cytoplasm, 4a.) Induction of macrophage apoptosis (Figure 2), each of which will be discussed here. A role for autophagy during the intracellular life cycle of *Francisella* has been suggested, however the role of this is currently uncertain (Figure 2, step 4b) [52].



**Figure 2: Intracellular life cycle of *Francisella*.**

The various stages of the *Francisella* intracellular life cycle are depicted. 1.) Entry by looping phagocytosis; 2.) Phagosomal escape; 3.) Extensive replication within the cytoplasm; 4a.) Induction of apoptosis and escape. 4b represents the recently described role for autophagy in the *Francisella* intracellular life cycle, however its true function has not yet been identified.

The first step in the intracellular lifecycle of *Francisella* is entry into a host cell. The bacteria are taken up by a novel mechanism that has been termed “pseudopod looping” [53]. Upon initial contact, the bacterium is surrounded by a long, looping pseudopod, which then fuses to the plasma membrane forming a large *Francisella* containing vacuole at the surface of the

macrophage. This process of uptake requires rearrangement of the actin cytoskeleton which is triggered by signaling through phosphatidylinositol 3 phosphokinase (PI3K) [53]. As the vacuole moves into the cell the size decreases, indicating that the initial spaciousness of the loop is not due to a large capsule surrounding the bacterium [53]. Looping phagocytosis occurs in response to live, heat killed, or protease-treated bacteria. Treatment of bacteria with periodate and lysine, which oxidizes and cross-links the surface carbohydrates, abolishes looping phagocytosis and bacteria are taken up through conventional phagocytosis [53].

The second step in *Francisella* intracellular survival is to escape into the cytoplasm. Under normal conditions, phagosomes containing extracellular particles interact with the compartments of the endosomal-lysosomal pathway, including early endosomes, late endosomes, and lysosomes [51]. In contrast, phagosomes containing live *Francisella* have been found to interact with the first two compartments only, never acquiring lysosomal markers [4]. Also, unlike phagosomes containing dead *Francisella*, these phagosomes are not acidified [4]. *Francisella* then escapes the phagosome, through an unidentified mechanism, to reside in the cytoplasm [4]. In human monocyte derived macrophages, this escape occurs within hours of infection, with 50% of bacteria in the cytoplasm by 6 hours and more than 80% phagosomal escape by 14 hours [4]. In contrast, bacteria appear to escape murine phagosomes much faster, with nearly all bacteria free in the cytoplasm within one hour of infection [52]. This escape process is known to require the *Francisella* virulence factors, intracellular growth locus C (*iglC*) and macrophage growth locus A (*mglA*) (discussed in detail below) [54,55]. At this point the bacteria are free to replicate in the cytoplasm. Studies have shown that *Francisella* has a doubling time of between three and five hours inside of macrophages, similar to its growth in synthetic culture media [56]. The bacteria then multiply, reaching high numbers in the

cytoplasm of macrophages [57]. Many genes have been shown to be vital to intracellular growth and survival in *Francisella* [58-61], these are discussed in detail below.

As intracellular bacterial numbers increase, a successful pathogen must eventually escape its host cell and move on to infect new cells. *Francisella* induction of apoptosis in host cells has been reported following extensive growth in the cytoplasm, however these studies have been carried out exclusively in cell lines [57,62]. Induction of apoptosis occurs within 18-24 hours following initial infection of a host cell [57,62,63]. While the mechanism responsible for *Francisella* induction of apoptosis remains to be fully elucidated, this process requires intracellular replication leading to the activation of host cellular caspases [62-64]. Once the bacteria have escaped the macrophage, they are free to infect other cells, effectively spreading throughout the host.

A topic of recent debate regarding the intracellular life cycle of *Francisella* is the role of autophagy late in infection of murine macrophages. Autophagy is a process by which cytoplasmic components, including proteins and organelles, are delivered to lysosomes for degradation [65]. This process has also been described as a mechanism for detection and removal of both bacteria and viruses [65,66]. A recent report by Checroun *et al.* demonstrated that *Francisella* moved back into a membrane bound vesicle (termed *Francisella* containing vacuole or FCV) following extensive replication within the cytoplasm [52]. These FCVs appear to fuse with lysosomes as they acquire the late endosomal/lysosomal marker LAMP-1. The formation of FCVs was found to be dependent on host cell autophagy, as multiple autophagy related molecules could be identified either on or in these vacuoles [52]. The mechanism involved in regulating this phenomenon during *Francisella* infection has yet to be identified, but it is speculated to be induced by the bacterium [52]. The formation of these vacuoles has not

been observed in studies that have examined human derived macrophages [4,53] leading to questions regarding the true role of these autophagy derived vacuoles in the course of natural *Francisella* infection.

With all of the data taken together, a model for *Francisella* intracellular survival and replication can be developed (Figure 2). The bacterium has evolved to utilize complex mechanisms for highly efficient intracellular replication, particularly within cells designed to eliminate pathogenic threats, such as macrophages and dendritic cells. The field has only begun to elucidate all of the mechanisms utilized by this bacterium for intracellular survival and growth.

#### **1.2.4 Virulence determinants of *Francisella***

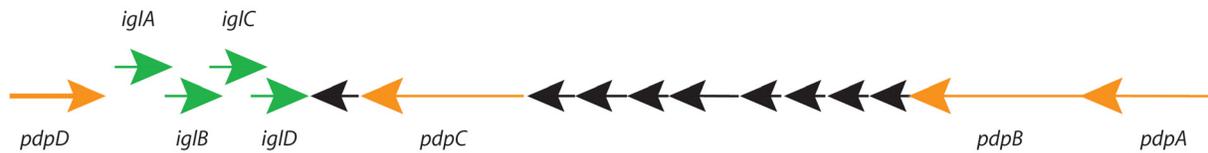
Although *F. tularensis* subsp. *tularensis* is one of the most virulent bacterial pathogens to be identified, few classical virulence factors have been identified in this microorganism [1]. One of the first virulence determinants identified in *Francisella* was the capsule. A capsule deficient mutant was developed by Sandstrom et al. in 1988 isolated as a “rough” colony identified following acridine orange treatment [67]. This capsule deficient mutant exhibited an increased sensitivity to serum killing and reduced proliferation in mice [67]. It is possible, however, that this mutant bore a defect in LPS biosynthesis, since LPS mutations can also elicit a rough colony morphology [68,69].

Until recently, research into the virulence mechanisms of *Francisella* has been severely hampered due to the lack of tools for genetic manipulation of this organism [70]. With the advent of new technologies, certain genes have been associated with virulence, particularly those involved in intracellular growth [71]. MglA (macrophage growth locus A) was one of the first

genes associated with virulence in *Francisella* [72]. In macrophages, this gene is required for intracellular growth and phagosomal escape [54,71]. *mglA* knockout *F. novicida* are highly attenuated, exhibiting a greater than  $10^5$  fold increase in LD<sub>50</sub> compared to wild-type [71]. MglA is an ortholog of the *E. coli* stringent starvation protein A (SspA). SspA is required for MglA association with *Francisella* RNA polymerase [11]. Through this association, MglA is able to regulate the expression of approximately 100 genes, most of which are proteins of unknown function [10].

One group of genes that is regulated by MglA is the *Francisella* Pathogenicity Island (Figure 3) [9,61]. Inactivation of any of the genes in this 30kbp segment of the genome leads to impaired growth in macrophages and reduced virulence in mice [9,61]. The more virulent subspecies (*tularensis* and *holarctica*) both contain two copies of the pathogenicity island, while *F. novicida* has only one [73,74]. Duplication and persistence of such a large stretch of genomic DNA is remarkable in an organism so prone to recombination events. Few of the genes in the pathogenicity island have been extensively studied. Two genes, intracellular growth locus (Igl) A and B, have been identified as components a possible type VI secretion system which interact with each other in the bacterial cytoplasm [75]. These two proteins are co-regulated, where bacteria lacking *iglA* exhibit a loss of expression of both proteins [75]. Another gene in the pathogenicity island, *iglD*, is also required for growth in macrophages. This protein is secreted into the macrophage cytoplasm during infection and is thought to play a role in altering the intracellular environment for bacterial growth [76]. The most studied gene within the *Francisella* pathogenicity island is *iglC*. IglC was originally identified as a 23 kDa bacterial protein that was up-regulated during growth within macrophages [77]. This protein is unique to the *Francisellae* with no homologs in any known bacteria. IglC has been knocked out in all

*Francisella* subspecies and is required for macrophage growth and virulence in each [70]. This protein is required for bacterial escape from the phagosome and is involved in the prevention of phagosome-lysosome fusion [54].



**Figure 3: Schematic representation of the genomic organization of the *Francisella* pathogenicity island.**

This genetic locus has been associated with virulence in all *Francisella* subspecies. The ORFs represented by black arrows are hypothetical proteins with no significant homology to other known bacterial proteins. The genes in the *iglABCD* loci (green arrows) appear to be organized in an operon.

Genes outside of the pathogenicity island have also been associated with virulence in *Francisella*. Recently, many more virulence associated genes have been identified using transposon mutagenesis [78] and targeted mutations [73,74], both relatively new technologies in the *Francisella* field. Early studies utilizing random DNA insertions to mutate the genome of *F. novicida* identified two candidate virulence factors that were associated with intramacrophage growth and murine virulence in *F. novicida* [79,80]. The first was a homolog of *minD*, is involved in resistance to oxidative killing in other organisms [79]. The second identified virulence determinant was *valAB*, an ABC transporter involved in LPS assembly that functions in serum resistance [80]. Utilizing transposon mutagenesis, Gray et al. identified five genes necessary for intracellular growth. Two of these genes were components of the pathogenicity island discussed above [61]. Other genes identified in this study included alanine racemase, ClpB protease, and an enzyme involved in purine biosynthesis [61]. Identification of genes

involved in various aspects of metabolism is common and expected, since the genome of this bacterium has evolved to contain the minimal sequence required for growth within macrophages [32]. Targeted mutagenesis has been used to identify many other genes involved in *Francisella* virulence including those involved in iron acquisition [12,81] and resistance to reactive oxygen and nitrogen intermediates [82,83].

Secretion of effector molecules is a virulence mechanism used by many bacterial species. Along with the putative type VI secretion system encoded by the *Francisella* pathogenicity island, genes with homology to type IV pilus systems and type II secretion systems have been identified in the genomes of *Francisella* species [84]. The role of these systems in virulence of *F. novicida* has been examined [85]. The secretion of six proteins through this system requires the presence of the pilin genes, *pilABC*. The role of the secreted proteins is currently unknown, though one of these, the peptidase PepO, appears to actually reduce the virulence of *F. novicida*. The role of this secretion system in more virulent strains of *Francisella* remains in question, especially since the effector PepO is not expressed in these strains [32]. Significant research is still necessary to fully understand the role of secretion systems and secreted effectors in the virulence of *Francisella*.

### 1.3 IMMUNE RESPONSE TO *FRANCISELLA*

The immune responses to the *Francisellae* have been intensely studied over the years. Many reports have shown that the host response to this organism is similar to that of other pathogens, leading many to utilize the *F. tularensis* live vaccine strain (LVS) as a model organism for intracellular pathogens [86]. However in recent years, researchers have begun to identify key differences which may be associated with this organism's relatively high virulence [87]. Many of the studies have been performed using strains that have minimal virulence in humans, including *F. tularensis* LVS and *F. novicida*, because tools are available for genetic manipulation of these bacteria. In addition, they are easier to work with since special containment is required to work with more virulent strains. As stated above, it is known that *F. tularensis* is able to survive and replicate within cells of the innate immune system, including macrophages and dendritic cells. Therefore, it is vital to understand how this pathogen is able to subvert the normal defenses of the body in order to fully understand its pathogenesis.

#### 1.3.1 Mechanisms of host cell recognition of a *Francisella* infection

Although *Francisella* is capable of replication in a variety of cell types, it is believed that the primary host cells infected by this bacterium are macrophages [4,53]. These cells, along with other innate immune cells including neutrophils and dendritic cells, are critical to successful host

defenses against pathogens as they are key mediators of pathogen recognition, inflammation, and bacterial killing. Cells of the innate immune system recognize pathogens using a variety of specific surface receptors, such as the Toll-like receptors (TLRs). Gram negative bacteria are composed of a variety of known TLR ligands including lipopolysaccharide (LPS), peptidoglycan (PG), lipoproteins, and unmethylated DNA. Recognition of bacterial LPS requires a complex consisting of TLR4, MD2, and CD14. While LPS is generally thought to be highly stimulatory, differences in structure can lead to altered levels of stimulation. *F. tularensis* LPS is known to stimulate macrophages only minimally, and does not activate cells through TLR4 or any other known TLR [88]. The LPS structure of *Francisella* is uncommon among bacterial species (detailed review in [89]). The LPS of *F. tularensis* LVS does not act as an agonist or antagonist of TLR4 and is not bound by LPS binding protein (LBP) [90]. With these data in mind, it is not surprising that mice lacking functional TLR4 do not exhibit an increased susceptibility to *Francisella* infection [91].

Alternatively, it is known that *F. tularensis* does stimulate cells through TLR2, which is commonly associated with bacterial PG. TLR2 is required for the stimulation of murine peritoneal macrophages to increase expression of cytokines including tumor necrosis factor (TNF) $\alpha$ , Interleukin (IL)-1 $\beta$ , and IL-12 p40 [92]. To date, the only identified TLR2 ligands in *Francisella* are the lipoproteins TUL4 and FTT1103, which are recognized by the heterodimer of TLR2/TLR1 [93]. However, other ligands must be present as *Francisella* also activates the TLR2/TLR6 heterodimer [94]. While TLR2 deficient mice survive intradermal infection with *F. tularensis* LVS, they are more susceptible to intranasal infection with LVS, indicating a role for this molecule in an effective immune response against respiratory tularemia [95].

While the role of specific TLRs remains somewhat unclear, other data show that downstream signaling systems are important to the host response to infection. The major signaling cascade following stimulation of a TLR is through an adaptor called myeloid differentiation factor (MyD)-88, thus linking this molecule to most TLR signaling that occurs within the cell. MyD88 knockout mice are highly susceptible to *Francisella* infection, even at normally sublethal doses, indicating that this molecule is vital for a successful host response to this infection [96]. Although MyD88 is also involved in IL-1 and IL-18 signaling, the contribution of signaling pathways induced by these cytokines appears minimal. Mice defective in IL-1 receptor or IL-18 exhibited only a minimal increase in susceptibility [96].

Since *Francisella* is an intracellular pathogen, a mechanism of pathogen recognition within the cytoplasm is likely to induce responses to this bacterium. NOD-like receptors (NLRs), including NOD1 and NOD2, are responsible for intracellular recognition of peptidoglycan and other microbial products [97]. Stimulation of these receptors leads to production of proinflammatory cytokines through signaling cascades similar to TLRs [97]. Activated NLRs recruit caspase-1 and other proteins to form a structure called the inflammasome. Assembly of this complex leads to the activation of caspase-1 and processing and secretion of IL-1 $\beta$  [98]. Both *F. novicida* and *F. tularensis* LVS induce caspase-1 activation in macrophages, monocytes, and dendritic cells, ultimately leading to IL-1 $\beta$  release [94,99,100]. These responses require the bacteria to be in the host cell cytoplasm, as mutants unable to reach the cytoplasm do not induce these responses [94,99,100]. The specific NLR protein(s) involved in *Francisella* recognition is currently unknown [101].

### 1.3.2 Role of Cytokines and Chemokines

Host cytokine production in response to *F. tularensis* has been intensely studied for years, leading to a significant body of information on the importance of these mediators of immune function during this infection. It has been reported these bacteria rapidly induce proinflammatory cytokines including interferon (IFN)- $\gamma$ , IL-12, IL-1 $\beta$  and TNF $\alpha$  following infection [92]. Levels of these cytokines present appear to differ based on the route of inoculation. Expression of IL-12, IFN $\gamma$ , and TNF $\alpha$  mRNA can be detected in the skin of mice within the first three days following subcutaneous infection [102]. Although high levels of systemic IFN $\gamma$  are detectable within one day following subcutaneous infection [96], this cytokine is not detected in lung homogenates until three days after intranasal inoculation [103].

These cytokines appear to play a critical role for the control of infection by the innate immune system. Mice deficient in T cell function, including *nu/nu* and  $\alpha/\beta$  T cell knockout mice, or those deficient in all lymphocytes (*scid* mice), are able to survive for 3-4 weeks before *Francisella* infection leads to death [104]. This early survival in lymphocyte-deficient mice depends on TNF $\alpha$  and IFN $\gamma$  because neutralizing antibodies against either cytokine significantly reduced survival [105]. IFN $\gamma$  knockout mice are extremely susceptible to LVS infection with even sublethal doses of bacteria leading to rapid mortality [96,105].

Another cytokine that has been implicated as an important player in the immune response to *Francisella* is IL-12. IL-12 is a heterodimeric cytokine consisting of two subunits p35 and p40 [106]. Macrophages and dendritic cells produce this cytokine following exposure to *F. tularensis* LVS [107,108]. Treatment of wild-type mice with intranasal IL-12 leads to protection against respiratory infection, however this protection requires intact IFN $\gamma$  signaling [103]. Mice deficient in IL-12 p40 production develop a chronic *Francisella* infection that is never fully

cleared. In contrast, mice lacking the p35 subunit of this cytokine appear fully immunocompetent and clear the infection at the same rate as wild-type mice [109]. This difference has lead researchers to examine the potential role of IL-23, a cytokine made up of the IL-12 p40 subunit and a unique p19 subunit, in a *Francisella* infection. *Francisella* induces IL-23 production from human monocytes leading to induction of IFN $\gamma$  from NK cells suggesting a role for this cytokine in the complete immune response to *Francisella* [110].

### **1.3.3 Role of B cells and specific antibodies in *Francisella* immunity**

The role of B cells in a *Francisella* infection appears to be complex as studies have produced variable results. It is clear that antibodies, including IgM, IgG, and IgA, are produced during natural *Francisella* infection and are detectable as early as two weeks post-infection [111]. These antibodies are long lasting and have been detected in patients after more than 10 years [111]. In murine infection models using *F. tularensis* LVS, IgM and IgG levels are detectable after 5 and 10 days, respectively [112]. Although it is commonly accepted that these antibodies are produced, the role they play in protection remains unclear. Studies have shown that passive transfer of immune serum protects mice against LVS challenge by decreasing bacterial burden in organs and increasing survival [112,113]. A portion of this protection may be the result of an increase in cytokine levels and neutrophil infiltration to the site of infection following transfer of specific antibodies [114]. There have been no reports of passive antibody transfer leading to protection against the more virulent type A strains of *Francisella* [87].

There appears to be a distinction between the contribution of antibodies and that of B cells during primary infection with *Francisella*. B cell knockout mice survive primary LVS infection similarly to wild type, exhibiting a delay in bacterial clearance and a less than 3 fold

increase in LD<sub>50</sub> observed [115]. Surprisingly, B cell knockout mice were severely impaired following secondary infection, exhibiting an LD<sub>50</sub> 100-fold lower than that of wild-type mice [115]. This defect was reconstituted by transfer of immune B cells, but not immune serum [115], indicating a role of B cells independent of antibody production. B cells also appear to provide a general, non-specific protection early in infection that is thought to be the result of IFN $\gamma$  production rather than specific antibody production [116].

Overall, it is clear that antibodies and B cells do offer protection from *Francisella* infection under certain circumstances; however, this protection appears to vary based on inoculating strain and route of infection. Further study will be required to elucidate the true contribution of these components of the immune response to this pathogen.

#### **1.3.4 Role of T cell mediated immunity in *Francisella* infection**

Although B cells and antibodies do appear to play a role in protection against *Francisella* infection, an optimal immune response leading to long term protection against this pathogen relies primarily on T cell responses [105]. T cell responses are detectable in humans within 2 weeks following infection with *Francisella* or vaccination using LVS [117,118]. The T cell responses against *F. tularensis* LVS have been well studied in the murine model [86]. As discussed above, T cell deficient mice are able to mount an initial, non-specific immune response that controls bacterial growth for the first weeks of infection [104]. Clearance of the infection, however, is clearly dependent on  $\alpha/\beta^+$  T cells, and mice lacking these cells succumb to infection within one month [119]. Survival from and clearance of infection can be mediated by either CD4<sup>+</sup> or CD8<sup>+</sup> T cells, as mice deficient in either type of T cell can resolve both primary and secondary LVS infection [119,120]. A double negative population of T cells (CD4<sup>-</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>)

that protects against *Francisella* infection has also been identified, though these cells are unable to elicit complete clearance of primary LVS infection [120,121]. These cells appear to function through the production of cytokines including IFN $\gamma$  and TNF $\alpha$ , though further research may identify other mechanisms of action for this unusual cell population [121,122]. Individually, each of these T cell subsets is capable of complete clearance of secondary LVS infections, though clearance occurs at a slower rate than in mice with all three subsets [119,120].

Unlike  $\alpha/\beta$  T cells, the role of  $\gamma/\delta$  restricted T cells in *Francisella* infection is less clear. Tularemia patients exhibit a rapid expansion of V $\gamma$ 9/V $\delta$ 2 T cells with a week of the onset of symptoms [123,124]. These T cell receptors are stimulated by nonpeptide phosphoesters termed “phosphoantigens,” which are produced by *F. tularensis* clinical isolates, LVS, and other pathogens, including *Mycobacterium tuberculosis* and *Plasmodium falciparum* [125,126]. Unfortunately, studies examining this subset of T cells are limited because mice do not have homologs to the human  $\gamma/\delta$  T cell receptors [87].

The mechanism of T cell action is currently unclear. IFN $\gamma$  from T cells likely activates macrophages to kill the intracellular *Francisella* [56]. IFN $\gamma$ -independent activation of macrophages by T cells has also been noted, though no specific mechanism has been identified to date [122]. As the specific host factors contributing to T cell immunity to *Francisella* are identified, the mechanisms involved will become clear. At this time, the specific T cell receptors, co-receptors, memory profiles, and MHC restriction of T cell responses to *Francisella* remain elusive. Identification of these factors along with specific mechanisms of T cell action would greatly increase our understanding of this pathogen.

### 1.3.5 Immune modulation by *Francisella*

While a large amount of information is available regarding the host immune responses to *Francisella*, studies have revealed that LVS has the ability to inhibit macrophage activation. Macrophages infected with LVS are unable to respond to TLR ligands such as *E. coli* LPS [127]. Once bacteria escape into the cytoplasm, *Francisella* infection leads to inhibition of NF $\kappa$ B activation and reduced phosphorylation of p38 and c-Jun leading to reduced levels of TNF $\alpha$  secretion [128]. It is known that a successful response to *Francisella* requires cytokine induction [105] therefore inhibition of host cell activation presumably provides an advantage to the bacterium.

#### 1.4 *FRANCISELLA* RESPONSE TO ENVIRONMENTAL SIGNALS

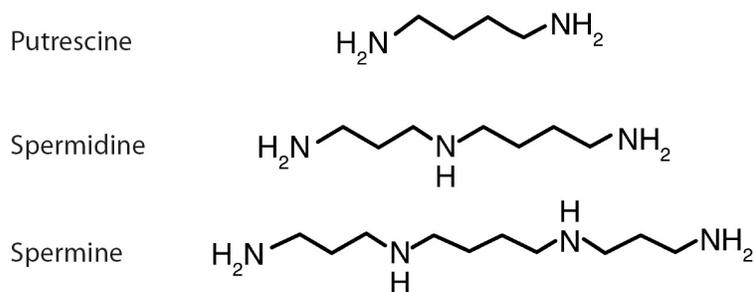
For a pathogen that can live in a wide range of environments and hosts, such as *Francisella*, it is important to develop mechanisms to sense environmental changes. Response of *Francisella* to some common bacterial signals has been shown. One signal encountered when moving from an environmental source or arthropod into a mammal is an increase in temperature. Not surprisingly, growth of *Francisella* at high temperature (40°C) leads to the induction of heat shock proteins [129]. A more complete analysis of gene expression changes when moving from an environmental temperature (26°C) to human body temperature (37°C) has demonstrated increased expression of genes involved in pathogenesis and intracellular growth (Joseph Horzempa, unpublished data). Another signal the bacterium encounters following infection of a mammalian host is iron limitation. Studies examining iron limitation in *Francisella* have shown the up-regulation of ~20 genes including members of the pathogenicity island in response to this signal. Another 20 genes were down-regulated following growth in low iron conditions, including gene encoding chaperone proteins and ribosomal proteins [12].

After *Francisella* infects a host, it will enter macrophages and other cells, moving into a new environment. The differences between the extracellular and intramacrophage environments lead to clear changes in *Francisella* gene and protein expression. The first identified member of the pathogenicity island, IglC, was identified as a 23 kilodalton protein exhibiting increased expression during intramacrophage growth [77]. Expression of IglC was also increased in response to oxidative stress, a condition the bacterium would encounter inside of a macrophage

[77]. In depth study of the *Francisella* response to this condition has lead to the identification of multiple proteins regulated by oxidative stress including heat shock and chaperone proteins and proteases among other proteins [129,130]. This response is controlled by MglA, a global regulator of virulence factors discussed above [131]. *Francisella* also encodes a mechanism to respond to the low glucose environment encountered in the macrophage cytoplasm [13]. Unidentified signals encountered inside of macrophages have also been associated with altered bacterial phenotypes. Although the mechanisms involved have not been identified, *Francisella* grown in macrophages are unable to stimulate cytokine secretion to the same level as bacteria grown in broth following subsequent infection [132]. Although researchers have begun to examine the interactions between *Francisella* and its hosts, it is clear that all the signals, and mechanisms utilized by the bacterium to respond to them, remain to be elucidated.

## 1.5 POLYAMINES

Polyamines, including spermine, spermidine, and putrescine, are low molecular weight, positively charged, polycationic compounds that are present in all living organisms (Figure 4). Prokaryotes produce primarily putrescine and spermidine, while spermine is also produced in eukaryotes [133]. These molecules are found at high concentrations intracellularly, often as high as 1mM [134]. Polyamines have been associated with a wide range of biological activities, including well documented interactions with RNA, DNA, and even some proteins [133-136]. The significance of these molecules in bacterial systems and pathogenesis will be discussed below.

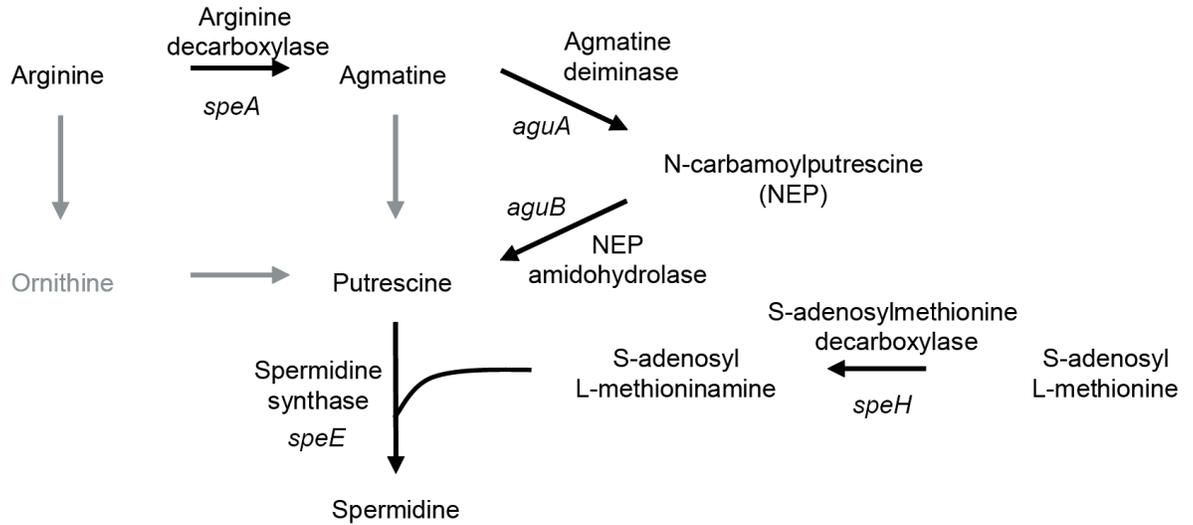


**Figure 4: Chemical structure of common polyamines.**

Putrescine and spermidine are produced by nearly all living organisms, while spermine is primarily found in eukaryotes.

### 1.5.1 Polyamine synthesis

Polyamine synthesis is carried out through a series of decarboxylases that act on the amino acids arginine and ornithine (Figure 5). In many bacteria, decarboxylation of ornithine, by ornithine decarboxylase (*speC*), leads directly to production of putrescine. *Francisella* genomes do not contain homologs of ornithine decarboxylase, so they must produce polyamines from arginine. Conversion of arginine to putrescine is a multistep process. First, arginine is converted to agmatine by arginine decarboxylase. Although many bacteria can convert agmatine directly to putrescine, the enzyme that catalyzes this reaction, agmatinase, is also absent from the *Francisella* genome. Instead *Francisella* utilizes a second pathway to perform this conversion through the intermediate N-carbamoylputrescine (see Figure 5). Using putrescine and S-adenosylmethionine as substrates, spermidine is produced by spermidine synthase [134].



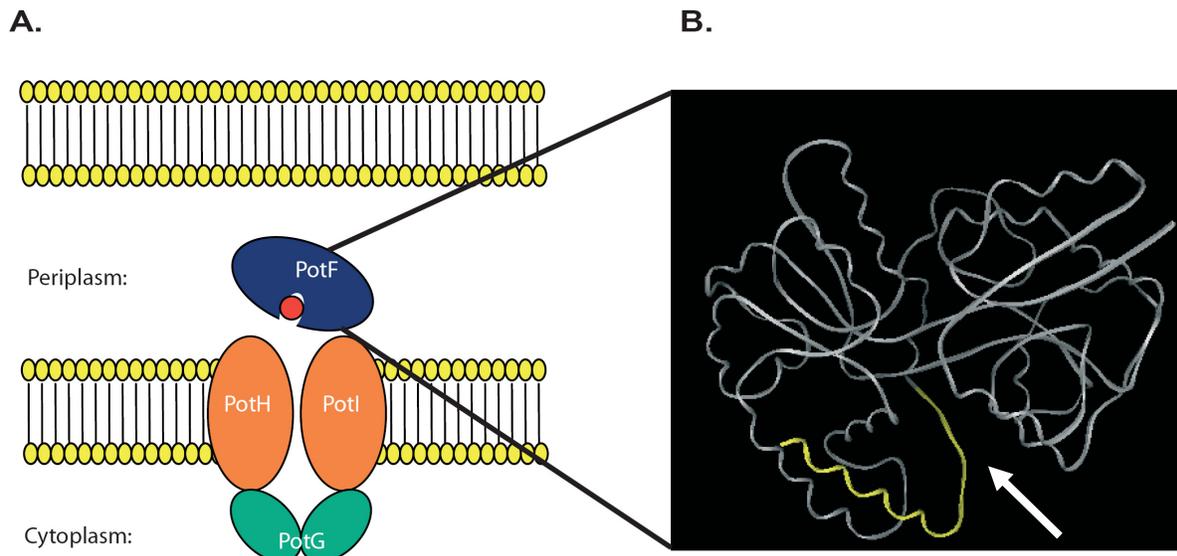
**Figure 5: Bacterial polyamine biosynthetic pathway.**

Biosynthetic pathway involved in the conversion of arginine to polyamines that occurs in bacteria. Spermidine is the endpoint of this pathway in prokaryotic organisms. *Francisella* is able to produce polyamines using this pathway, however the steps represented in gray are not present in the *Francisella* genome. *Francisella* specific gene designations for the various proteins are listed.

### 1.5.2 Transport of polyamines

Not all polyamine content in a bacterium must be synthesized. All known bacteria, including *Francisella*, encode polyamine transport systems. Polyamine uptake in *E. coli* exhibits a preference for smaller molecules with rates of uptake being putrescine > spermidine > spermine [137,138]. Most bacteria encode two polyamine transport (pot) systems. These have been identified as the spermidine/spermine uptake system, encoded by *potABCD* and the putrescine uptake system, encoded by *potFGHI* (Figure 6a) [137,138]. Each of the polyamine uptake systems is an ABC transporter formed by four gene products. The components of these transport systems include a periplasmic binding proteins (*potD* or *potF*), two channel forming

transmembrane proteins (*potAB* or *potHI*), and an intracellular ATP binding protein (*potC* or *potG*) [137,138]. The genomes of *Francisella* subspecies only contain one of these systems. PotD/ PotF (structure shown in Figure 6b) function to bind polyamines in the bacterial periplasm and sequester these molecules to the other components of the transporter. The transmembrane proteins and ATPase then mediate the transport of polyamines across the membrane in an energy dependent manner [137,138]. In *F. tularensis* spp. *tularensis* this system has been annotated as *potABCD*, while the same system in *F. tularensis* spp. *holarctica* LVS has been annotated as *potFGHI* [32]. The actual role and specificity of these transport systems in *Francisella* are currently unknown as they have not been studied in this organism. A potential role in sensing and responding to polyamines has been suggested for homologs of these transporters [139]. Other ABC transporters, including those for maltose and glucose in *E. coli*, are capable of controlling transcription in response to binding these molecules [140]. In these systems, activation of the ATPase leads to either binding or release of specific transcription factors following activation of the transporter [140].



**Figure 6: Polyamine transport in bacteria.**

(A.) Schematic representation of the *Francisella* polyamine transporter. This ABC transport system consists of a periplasmic binding protein (PotF), a membrane channel made up of two proteins (PotH and PotI), and an ATPase (PotG). (B.) Structural model of the periplasmic binding protein, PotF. Binding pocket indicated with an arrow.

### 1.5.3 Function of polyamines in bacterial systems

As stated above, polyamines are vital to the survival and growth of most bacterial species, with strains deficient in polyamine production exhibiting a 70% decrease in growth rate (Tabor 1985). Many functions have been associated with these compounds, but perhaps the most characterized is the interaction between polyamines and nucleic acids [133-136]. In *E. coli*, spermidine is primarily found in association with nucleic acids with 90% bound to RNA and about 5 % bound

to DNA. Only approximately 4% of this polyamine is found free in the bacterium. Similarly, putrescine interacts with RNA and DNA (48% and 9%, respectively) with 39% free in the cytoplasm [135].

Polyamines are thought to play a variety of roles through interactions with DNA. The ability of polyamines to bind and stabilize bent DNA has been demonstrated and is thought to play a role in DNA condensation [141]. This mechanism could regulate multiple functions through DNA binding proteins, such as transcription factors, which may recognize and bind to bends in DNA [134]. Polyamine interaction appears to be vital for the thermophile *Thermus thermophilus*. This bacterium synthesizes unusual polyamines, including long chain polyamines and branched polyamines [142]. These molecules alter the melting temperature of DNA in this organism and are required for growth at high temperatures (>75°C) [142].

The presence of polyamines leads to significant increases in transcription [134] and RNA stability [143]. In fact, polyamines have been shown to regulate gene expression changes in *E. coli*, with more than 600 genes being regulated solely by the addition or removal of putrescine [144]. The mechanism of RNA stabilization by polyamines is thought to be through stabilization of RNA secondary structure [135]. Such stabilization would have dramatic effects on protein synthesis. In cell free systems, addition of polyamines has been shown to increase the optimal rates of protein synthesis [135]. Further enhancement of protein synthesis by polyamines has been described through their interaction with ribosomal components [145-147].

Many other roles of polyamines in bacterial cells are beginning to be elucidated. Polyamines have been shown to function in the oxidative stress response in *E. coli* through the up-regulation of *oxyR* and *katG* [148]. These molecules have also been associated with the ability of bacteria to flourish in high temperature environments [142]. Interactions of

polyamines with the bacterial cell wall have also been described and appear to lead to enhance bacterial survival and general cellular stability [134].

#### **1.5.4 Role of polyamines in bacterial virulence**

Polyamines have been associated with mechanisms of virulence in various microorganisms. One major role of these molecules in pathogens is iron scavenging. Polyamines form the backbone of many siderophores, iron binding molecules known to be important for growth in mammalian systems [134]. Siderophores in *Bordetella pertussis*, *Vibrio cholerae*, and *F. tularensis* are produced from a polyamine backbone [81,149,150].

Direct involvement of polyamines in virulence has been demonstrated in *Streptococcus pneumoniae*. The periplasmic binding protein (*potD*) of the polyamine transporter is required for full virulence in this pathogen, with disruption of this gene leading to a significant decrease in virulence in murine infection models [151]. Polyamines have also been associated with biofilm formation, a virulence mechanism utilized by many microorganisms. In the plague causing bacterium, *Yersinia pestis*, polyamines produced by the bacterium are required for effective formation of biofilms [152]. Similarly, the ability to sense polyamines has been associated with biofilm formation in *V. cholerae*. The formation of biofilms in this intestinal pathogen requires the expression of a predicted polyamine sensor, NspS, which has homology to the *potD* and *potF* genes described above [139].

## 1.6 STATEMENT OF THE PROBLEM ADDRESSED IN THIS THESIS

The goal of this study was to identify environmental signals affecting the pathogenesis of *F. tularensis* and the changes induced in the bacterium by these signals. *Francisella* exhibit a remarkable ability to adapt to a wide range of hosts, including many mammalian and arthropod species. The bacterium must have the ability to sense its various environments and alter gene and protein expression accordingly. The mechanisms involved in this adaptation have only begun to be elucidated. The work detailed in this thesis addresses these ideas, with the goal of understanding the interactions that occur between LVS and host macrophages during *Francisella* infection. We first characterized a variant of *F. tularensis* LVS that differed in its ability to stimulate macrophages compared to wild-type LVS. The variant arose from an altered method of culturing the bacterium, indicating that signals encountered during routine growth in broth culture can influence bacterial phenotypes. Similarly, the macrophage response to LVS grown in different media was examined and found to be vastly different. By examining the components of these media, we have identified the polyamine spermine as the molecule responsible for inducing a macrophage inhibitory phenotype in LVS. The response of *F. tularensis* LVS to spermine was characterized and examined as a potential mechanism of bacterial recognition of the host intracellular environment.

- To identify differences between wild-type LVS and a variant that induces macrophage activation.

- To characterize altered phenotypes and gene expression patterns induced in LVS under different growth conditions.
- To identify specific signal(s) responsible for the phenotypic variations observed.
- To characterize the bacterial response to spermine and the role of this response in pathogenesis.

## 2.0 CHAPTER 1: MODULATION OF VIRULENCE FACTORS IN *FRANCISELLA TULARENSIS* DETERMINES HUMAN MACROPHAGE RESPONSES.<sup>1</sup>

### 2.1 ABSTRACT:

*Francisella tularensis*, the causative agent of tularemia and Category A biodefense agent, is known to replicate within host macrophages, though the pathogenesis of this organism is incompletely understood. We have isolated a variant of *F. tularensis* Live Vaccine Strain (LVS) based on colony morphology and its effect on macrophages. Human monocyte-derived macrophages produced more tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, and IL-12 p40 following exposure to the variant, designated the activating variant (ACV). The immunoreactivity of the lipopolysaccharide (LPS) from both LVS and ACV was comparable to the previously described blue variant and was distinct from the gray variant of LVS. We found, however, the soluble protein fractions of LVS and ACV differed. Further investigation using two-dimensional gel electrophoresis demonstrated higher levels of several proteins in the parental LVS isolate. The differentially-expressed proteins featured several associated with virulence in *F. tularensis* and other pathogens, including intracellular growth locus C (IglC), a  $\sigma^{54}$  modulation protein family member (YhbH), and aconitase. ACV reverted to the LVS

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<sup>1</sup> Work in this Chapter was published in *Microbial Pathogenesis*, Vol. 42, No. 5-6, pp. 204-214. May-June 2007. © 2007 by Elsevier.

phenotype, indicated by low cytokine induction and high IglC expression, after growth in a chemically-defined media. These data provide evidence that the levels of virulence factors in *F. tularensis* are modulated based on culture conditions and that this modulation impacts host responses. This work provides a basis for investigation of *Francisella* virulence factor regulation and the identification of additional factors, co-regulated with IglC, that affect macrophage response.

## 2.2 INTRODUCTION:

*Francisella tularensis*, the causative agent of tularemia, is a facultative intracellular bacterium, known to replicate in macrophages [1]. *F. tularensis* subspecies *tularensis* is highly infectious, with intradermal or inhalation routes of infections requiring only about 10 organisms to cause serious disease [1]. If untreated, death rates from pulmonary tularemia can be as high as 60% [2]. These factors have led to concerns over the potential use of *F. tularensis* as a biological weapon, leading to its classification as a Category A biodefense agent [2]. Although there is a vaccine for this disease, the live vaccine strain (LVS) is no longer licensed for use because of both its inability to induce complete protection and its unknown mechanism of attenuation [153]. LVS, which was derived from *F. tularensis* subspecies *holarctica*, exhibits minimal virulence in humans, but has been used extensively to study tularemia due to its virulence in murine models [86].

Macrophages are important components of the innate immune system, functioning to engulf and kill microorganisms. Some bacteria, including *Francisella*, survive and replicate within these cells [4], emphasizing the value of examining the interaction between macrophages and *Francisella*. It is also important to understand *Francisella* in the context of human infection. Though these organisms have been intensely studied in the murine model [86], human cell responses to these pathogens is less well studied [154,155]. Similarly, investigation into the cytokine response these organisms elicit from human macrophages has been limited [156].

Several important virulence determinants have been identified in *F. tularensis*. LPS [1] and capsule [67] contribute to virulence because the LPS induces only low levels of proinflammatory cytokines, IL-1 $\beta$  and TNF $\alpha$ , while the capsule improves resistance to complement. In addition, specific proteins are required for intramacrophage growth by *F. novicida* [61] and *F. tularensis* LVS [9], including the *iglABCD* operon [54,55,64,71,73,74,77] and the *pdpA* and *pdpD* genes [9] contained within the *F. tularensis* pathogenicity island [9]. Nevertheless, the functions of these proteins and the overall pathogenesis of *F. tularensis* remain incompletely understood.

In conducting studies of host cell responses to *F. tularensis* LVS, we have isolated a variant that differed from the parental LVS strain in colony morphology and in the ability to elicit cytokines from human macrophages. This variant appears distinct from previously identified variants [157]. Our closely related *F. tularensis* isolates were used to identify putative virulence factors that may influence macrophage responsiveness. Moreover, we show that culture conditions modulate the level of virulence factor expression in this organism. Comparing the LVS and ACV isolates will facilitate identification of putative *Francisella* virulence factors that are co-regulated with IglC.

## 2.3 MATERIALS AND METHODS:

### Francisella strains and cultivation:

*F. tularensis* LVS and *F. novicida* (U112) were kindly provided by Dr. Karen Elkins (U.S. Food and Drug Administration). Fresh Mueller-Hinton (MH) broth supplemented with 0.1% glucose, 0.025% ferric pyrophosphate (Sigma), and Isovitalex was used to grow stocks of LVS. For reversion experiments, bacteria were grown in a chemically defined media (CDM) described by Chamberlain in 1965 [158]. Low density broth cultures were pre-incubated at 37°C with 5% CO<sub>2</sub>, then grown at 37°C with agitation until the cultures reached OD<sub>600</sub> ≤ 0.2, approximately 8-10 hours. The activating variant (ACV) was isolated from, and maintained in, cultures grown in MH broth to higher densities (OD<sub>600</sub> ≥ 1.0). To induce reversion of ACV to LVS, bacteria were cultured in CDM overnight, to an OD<sub>600</sub> ≥ 1.0. Bacteria were then centrifuged and either used fresh, or resuspended in PBS + 20% glycerol and stored at -80°C until needed for infection. Experiments using actively growing *Francisella* cultures yielded results comparable to those in which macrophages were exposed to frozen stocks. For RNA and protein isolations, *F. novicida* and LVS isolates were grown on chocolate II agar at 37°C and 5% CO<sub>2</sub> for one or three days, respectively, and harvested as indicated below. All bacterial growth media and supplements used in these experiments were purchased from BD Biosciences, unless otherwise stated.

### Macrophage culture and infection:

Human macrophages were differentiated from monocytes by *in vitro* culture. Elutriated human monocytes (> 95% purity) were purchased from Advanced Biotechnologies Inc. and cultured at a density of approximately  $2.5 \times 10^7$  in 60mm culture dishes for seven days at 37°C with 5% CO<sub>2</sub> in 9ml of DMEM (Invitrogen) containing 20% FCS (Invitrogen), and 10% human serum (Gemini Biosciences). Some experiments were performed using monocytes isolated from human buffy coats of blood donations (Central Blood Bank, Pittsburgh). These cells were purified using Ficoll gradients (Amersham Biosciences) to isolate PBMCs, Optiprep gradients (Axis-Shield) to enrich for monocytes, and negative selection magnetic column separation (Miltenyi Inc.) or panning on plastic to further purify monocytes (final purity > 95% based on microscopy). These cells were then cultured similarly to the elutriated monocytes. On day seven, macrophages were removed from the culture dish using a lidocaine/EDTA solution (5 mM EDTA and 4 mg/ml lidocaine in PBS pH 7.2). Cells were washed and resuspended in DMEM containing 1% human serum prior to plating onto Primaria 96-well plates (BD-Falcon) at a density of  $5.0 \times 10^4$  –  $1.0 \times 10^5$  cells per well. Unless otherwise indicated, macrophages were exposed to bacteria at a multiplicity of infection (MOI) of approximately 10 for 24 hours before supernatants were collected. For TLR inhibition studies, macrophages were incubated with either LVS or ACV for four hours at an MOI of 500. The high MOI was used to increase the percent of macrophages (> 70%) infected following the short incubation. Macrophages were incubated with HBSS containing gentamicin (20 µg/ml) for 15 minutes to kill extracellular bacteria then washed three times with warm HBSS. *E. coli* LPS (1µg/ml) was then added to the macrophages for 20 hours before collection of supernatants. All use of human-derived cells was approved by the University of Pittsburgh Institutional Review Board.

### ELISA analysis:

Macrophage supernatants were harvested 24 hours after introduction of bacteria and were tested by ELISA analysis to measure cytokine levels. TNF $\alpha$  was measured using a matched antibody pair (R&D Systems) and IL-1 $\beta$ , IL-6, IL-12 p40 were measured using DuoSets (R&D Systems). Following the addition of TMB substrate solution (Dako) and measurement of optical density using a Molecular Dynamics M2 plate reader, cytokine levels were calculated from a standard curve. The limits of detection for the ELISAs were: TNF $\alpha$  – 15pg/ml, IL-1 $\beta$  – 15pg/ml, IL-6 – 15pg/ml, and IL-12 p40 – 10pg/ml.

### Macrophage viability:

Macrophage viability was examined following a 24 hour incubation with either media only or bacteria at an MOI of 10. At the end of the incubation, supernatants were harvested and tested for the presence of lactate dehydrogenase (LDH) using the Cytotoxicity Detection Kit<sup>PLUS</sup> (Roche) according to manufacturer's protocol. Percent lysis was calculated by comparing samples to maximal LDH release from macrophages treated with the lysis buffer provided in the kit. Data are presented as % maximal LDH release where % max LDH = (sample LDH – background)/(maximum LDH – background) \*100). Following the removal of supernatants from the macrophage cultures, the cells were tested immediately for staining with Invitrogen's Live/Dead staining kit according to manufacturer's protocol. SYTO9 (live) and propidium iodide (dead) staining was measured using a Molecular Dynamics M2 plate reader at the following excitation and emission wavelengths: SYTO9 480/500nm, propidium iodide 490/635nm.

### Protein Electrophoresis and Immunoblotting:

*F. tularensis* LVS, *F. tularensis* LVS - ACV, and *F. novicida* were harvested from chocolate II agar plates, suspended in 3ml of PBS, and lysed by French press using two passes at 18,000 lb/in<sup>2</sup>. These whole cell lysates were then fractionated into membrane-associated and soluble proteins by ultracentrifugation at 314,000 x g for one hour as described [159]. For single dimension gels, 10µg of total protein was solubilized in 2x Lamellae buffer and loaded in each well. Proteins were separated through a 4% acrylamide stacking and 12.5% acrylamide resolving gel at 30mA prior to staining with Silver Stain Plus (Bio-Rad). Protein concentrations in whole cell lysates and soluble fractions were determined by D<sub>C</sub> Protein assay (Bio-Rad), while membrane protein concentrations were determined by modified Lowry using BSA as a standard [160].

For LPS western blots, cell lysates from the two isolates (LVS and ACV) were treated with proteinase K before being separated on a 4-12% Bis-Tris gradient gel (Invitrogen). Separated material was transferred to PVDF membrane using XCell II<sup>TM</sup> Blot Modules (Invitrogen), according to manufacturer's protocol. Following transfer and blocking with 5% (wt/vol) non-fat dry milk in TBST, membranes were probed with either a mouse anti-*F. tularensis* LPS monoclonal antibody or a mouse anti-*F. novicida* (#8.2) monoclonal antibody (both from Immuno-Precise Antibodies Ltd.). Anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Invitrogen) was used for detection and fluorescence was visualized on a Typhoon 9410 (Amersham Biosciences).

For two-dimensional gel electrophoreses, soluble protein fractions were isolated as described above and then precipitated by 80% acetone (v/v) and 10% TCA (v/v) and pelleted by centrifugation (16,000 x g, 20 min, 4°C). Protein pellets were suspended in C<sub>4</sub>TT<sub>3-10</sub> solubilizing

solution [161] and incubated overnight at 23°C with gentle agitation. Soluble material was clarified by ultracentrifugation (314,000 x g, 1 hr, 23°C), and 200µg of protein was loaded onto 13 cm, pH 3 to 10 linear IPG strips and focused in the first dimension for 82,000 Vh using the IPGphor system (Amersham Biosciences) (running conditions: 500V for 1hour, 1500V for 1 hour, 8000V for 80,000 Vh). Focused strips were stored at -80°C until separated in the second dimension by SDS-PAGE. Proteins were separated in the second dimension by 12.5% acrylamide gel for 3-4 hours at 35mA per gel prior to staining with Silver Stain Plus (Bio-Rad).

IgIc immunoblots were performed with either whole cell lysates used for two-dimensional gels (1 µg total protein per lane) or from overnight bacterial broth cultures. For overnight cultures, 1.0 ml of was pelleted, washed, and resuspended in 100 µl of LDS loading buffer (Invitrogen), boiled for 5 minutes and sonicated. One µl of diluted (1:10) whole cell lysate were separated on a 12% acrylamide gel and transferred as stated above. Membranes were probed with a mouse anti-IgIc monoclonal antibody (clone 10D12, 1:20 dilution of hybridoma supernatant, Immuno-Precise Antibodies Ltd.) followed by an anti-mouse IgG antibody conjugated to HRP (1:15000, Sigma). Proteins were visualized using ECL chemiluminescent reagent (Amersham Biosciences) and exposure to film. Fold difference in protein amounts were calculated by comparing integrated density values (IDV) a BioRad Gel Doc system and Quantity One software (V4.4.0) ( $IDV = \sum(\text{each pixel value} - \text{background pixel value})$ ). Immunoblots were re-probed with a rabbit anti-hsp70 polyclonal antibody (1:750, GeneTex, Inc.) followed by an anti-rabbit IgG antibody conjugated to HRP (1:15000, Sigma), to examine protein load. Proteins were visualized using ECL and exposure to film as detailed above.

MALDI-TOF analysis:

Protein spots of interest were picked manually (1.0 mm to 3.0 mm in diameter) and rinsed in 400  $\mu$ l distilled water. Silver ions were removed by adding 50  $\mu$ l fresh destaining solution (15 mM potassium ferricyanide and 50 mM thiosulfate in distilled water) (Invitrogen, Carlsbad, CA) to each spot and incubating for 20 min. Samples were then rinsed twice with 500  $\mu$ l distilled water and equilibrated with 100 mM ammonium bicarbonate (Fisher) for 20 min. Samples were rinsed 3 x in 400  $\mu$ l of 50% acetonitrile (Fisher) in 100 mM ammonium bicarbonate (23°C, 15 min) and dehydrated in 400  $\mu$ l of 100% acetonitrile (23°C, 10 min). Samples were digested in situ with 200-300 ng trypsin (Sigma) (14 hours, 37°C) and peptides were extracted twice with 50  $\mu$ l of 50% acetonitrile, 2.5% TFA (Fisher) in distilled water and dried using a CentriVap Speed Vacuum. Extracted, dried peptides were mixed with 5  $\mu$ l of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) and 0.5  $\mu$ l was spotted onto the target for MALDI-TOF analysis. MALDI-TOF was performed using the 4700 Proteomics Analyzer (Applied BioSystems, Foster City, CA). The 4700 Proteomics Analyzer was primarily calibrated using the CalMix from ABI with the following instruments settings: *i*) minimum S/N of 10, *ii*) mass tolerance of +/- 2 m/z, *iii*) minimum peaks to match of 6, *iv*) maximum outlier error of 5 ppm, and *v*) a laser intensity of 3900. Mass spectra were individually calibrated using internal trypsin peaks with Data Explorer software available from ABI. Proteins were identified using ProteinProspector (University of California, San Francisco; <http://prospector.ucsf.edu/>) set to a mass accuracy of +/- 50 ppm to compare unknown mass fingerprints to those of known proteins in the NCBI database using a species-specific filter for *F. tularensis*.

## 2.4 RESULTS:

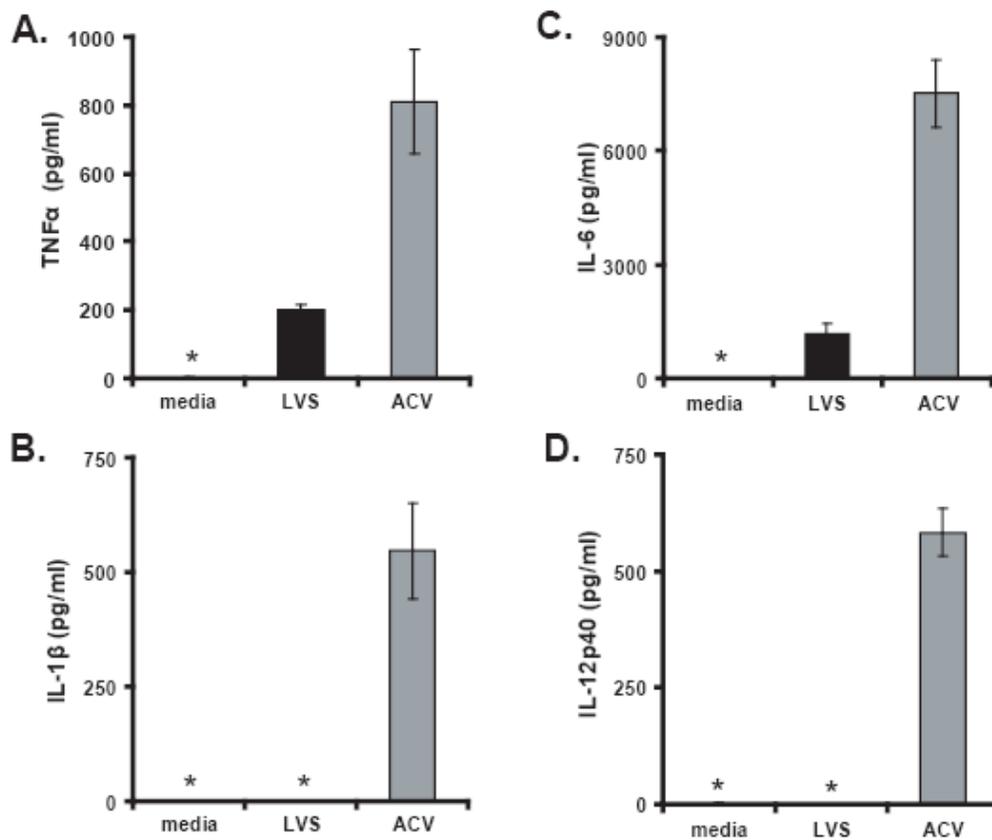
### 2.4.1 Isolation of a novel LVS variant.

While examining macrophage responses to *F. tularensis* LVS, we identified a variation of LVS colony morphology. The variant emerged from LVS cultures grown to an  $OD_{600} \geq 1.0$  in Mueller-Hinton (MH) broth. Colonies of LVS had a bright white appearance, while the variant colonies exhibited a more dull color on 5% sheep blood tryptic soy agar plates. Once generated, the phenotypes of these isolates were stable on sub-culturing in MH broth (LVS parent isolate was stable to  $OD_{600} < 0.25$ ) or chocolate II agar plates. The LVS variant reproducibly arose from high density cultures and individual colonies derived from those cultures had a similar appearance on tryptic soy agar plates with 5% sheep blood. Re-culturing the variant at low densities in MH broth failed to revert the variant to the original LVS appearance (data not shown).

### 2.4.2 Human macrophage response to *Francisella* isolates.

Since previous reports have shown blue and gray LVS variants induce different responses from rat macrophages [157], we tested our LVS and variant for their ability to activate macrophages. LVS and our colony variant were added to cultures of human monocyte-derived macrophages and supernatants were harvested after 24 hours. We found that macrophages

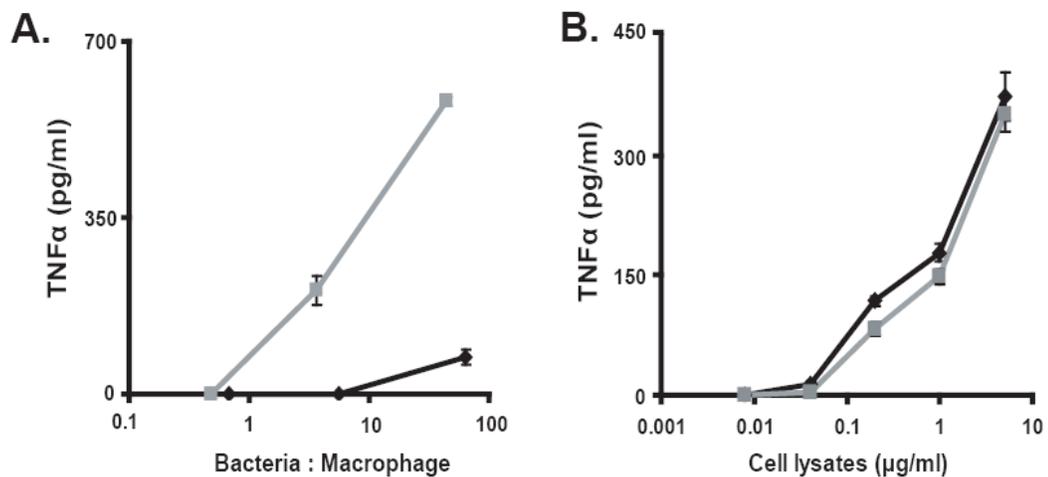
produced higher levels of TNF $\alpha$ , IL-1 $\beta$ , IL-6, and IL-12 p40 in response to the variant compared to the parental LVS (Figure 7). In fact, there was no detectable IL-1 $\beta$  or IL-12 p40 induced by the parental LVS (Figure 7B, D). Because the variant induced higher levels of proinflammatory cytokines, we termed this an activating variant (ACV) of LVS.



**Figure 7: Proinflammatory cytokine production by human monocyte derived macrophages following exposure to *F. tularensis* LVS and ACV.**

Macrophages were cultured with either LVS or ACV at an MOI of 10 for 24 hours. Levels of TNF $\alpha$  (A.), IL-1 $\beta$  (B.), IL-6 (C.), and IL-12 p40 (D.) were measured in supernatants by ELISA. Data are mean  $\pm$  SD of triplicate wells within one experiment. Data are representative of 5 individual experiments with different donors. \* indicates cytokine levels below detectable limits of the ELISA.

Since some published reports have used higher doses of organisms during *in vitro* infection experiments [62,63], it was possible that the low macrophage response to LVS observed in our experiments was due to an inadequate bacterial inoculum. To test this possibility, we examined the macrophage response to different numbers of bacteria. Macrophages were co-cultured for 24 hours with a range of MOIs. Even at an MOI of 100, LVS elicited substantially less TNF $\alpha$  than ACV (Figure 8A). Forty to sixty percent of macrophages exposed to MOIs in this range for four hours were associated with bacteria by fluorescence microscopy. Therefore, LVS failed to induce cytokines even though there was a demonstrable interaction between the bacteria and the macrophages.

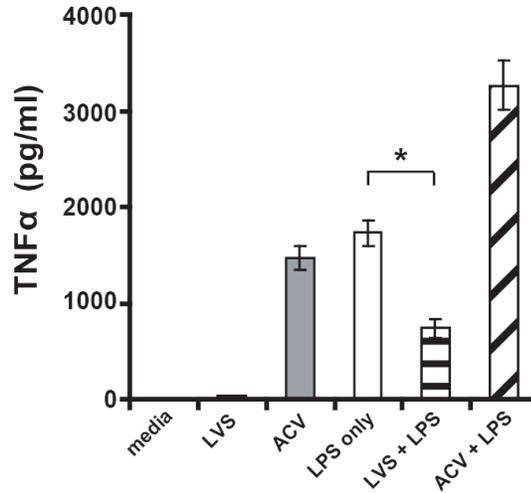


**Figure 8: Macrophage response to live bacteria and bacterial lysates.**

(A.) TNF $\alpha$  production by human monocyte derived macrophage exposed to a range of MOIs of LVS ( $\blacklozenge$ , black lines) or ACV ( $\blacksquare$ , gray lines) for 24 hours. Data are mean  $\pm$  SD of triplicate wells within one experiment. (B.) Decreased cytokine response to LVS requires live, intact bacteria. Proteins lysates from each variant stimulate macrophage TNF $\alpha$  production. Data are mean  $\pm$  SD of triplicate wells within one experiment. Cytokine levels were measured by ELISA and similar results were seen in macrophages from 3 separate donors.

It was possible that ACV expressed ligands for Toll-like receptors (TLR), while LVS did not, or that such ligands were hidden from macrophage recognition in LVS. To address these possibilities, we compared whole cell lysates from LVS and ACV for their ability to activate human macrophages. Lysates from both isolates elicited comparable dose-response curves of TNF $\alpha$  production from human macrophages (Figure 8B). Comparable levels of cytokine secretion were also observed when macrophages were exposed to either isolate killed by freeze-thaw (data not shown). These data demonstrate that components of LVS are capable of activating macrophages, suggesting these components were masked in live bacteria or that live LVS actively inhibited host responses. In contrast, ACV appears to have lost this ability to mask its stimulatory components or inhibit host responses.

Wild type *F. tularensis* LVS inhibits normal TLR4 signaling [127] and we speculated that ACV lost this property, providing an explanation for the ability of ACV to induce higher levels of cytokines from macrophages (Figure 7). To test this, macrophages were cultured for 4 hours with LVS or ACV such that 70% of the macrophages contained bacteria on immunofluorescence staining (data not shown). The macrophage cultures were then washed after the 4 hour incubation to remove extracellular bacteria, and *E. coli* LPS was added to stimulate TNF $\alpha$  production. As we have seen previously (Figure 7A), LVS elicited low levels of TNF $\alpha$  while ACV induced high levels of the cytokine (Figure 9). As expected, LPS alone also induced high levels of TNF $\alpha$  (Figure 9). Macrophages pre-incubated with ACV and then cultured with LPS produced the highest levels of TNF $\alpha$ . In contrast, pre-incubation with LVS significantly inhibited TNF $\alpha$  induction by LPS (Figure 9). These results suggested inhibition of TLR signaling was the mechanism for low cytokine responses to LVS compared to ACV.



**Figure 9: Inhibition of macrophage TLR signaling by LVS.**

Macrophages were incubated with either media, LVS, or ACV for 4 hours (MOI = 500), washed, and treated with LPS for 20 hours. Following incubation, supernatants were harvested and levels of TNF $\alpha$  were measured using ELISA. Data are mean  $\pm$  SD of triplicate wells within one experiment and are representative of experiments performed on two separate donors. \* denotes  $p < 0.002$ .

*F. tularensis* has been shown to induce host cell death [62,63], raising the possibility that LVS caused rapid macrophage death, thereby preventing cytokine production by the host cells. We assessed host cell viability after exposure to the LVS isolates by two strategies. First, we measured release of lactate dehydrogenase (LDH) from the cytoplasm of macrophages after incubation with LVS or ACV for 24 hours, as has been done previously [156]. No differences in LDH release were observed between macrophages cultured with LVS and ACV ( $p = 0.7$  by t-test) (Table 2). The second strategy used fluorescent stains that distinguish between live and dead cells (Live/Dead staining kit, Invitrogen). The results were similar to those obtained measuring LDH release in that no significant differences in macrophage viability were seen following exposure to LVS or ACV ( $p = 0.88$  by t-test) (Table 2). LVS and ACV at high MOI, such as that used in the TLR inhibition experiments (Figure 9), did not result in appreciable

differences in macrophage viability (data not shown). These data suggested that macrophage death was unlikely to account for the differences in cytokine production following exposure to the LVS isolates.

**Table 2: Macrophage viability following incubation with LVS isolates.**

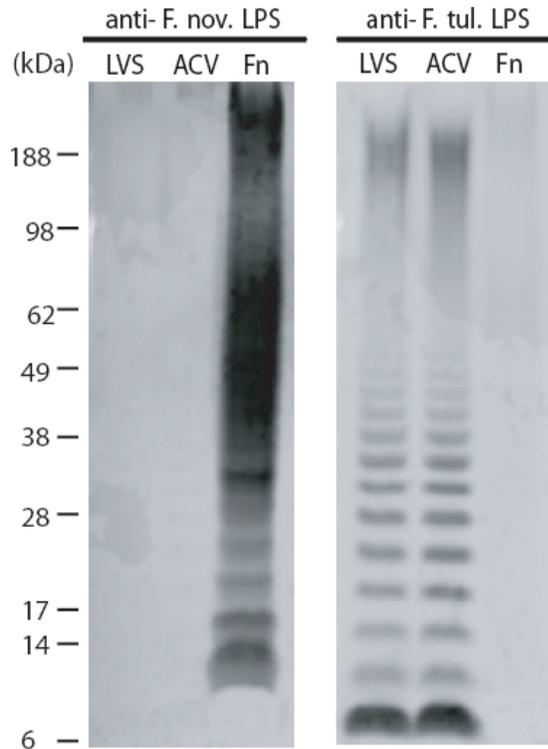
Sample	% max LDH release <sup>a,b</sup>		Live (SYTO9)/Dead (PI) <sup>a,b</sup>	
	mean	std. dev.	mean	std. dev.
Media	12.25	± 1.73	18.65	± 0.70
LVS	14.02	± 2.19	17.7	± 0.21
ACV	13.26	± 3.54	17.73	± 0.23

<sup>a</sup> Data are representative of three experiments on separate donors.

<sup>b</sup> Other experiments showed modest elevations of LDH after bacterial culture, though no differences were seen between the treatment groups.

### 2.4.3 Molecular characterization of LVS isolates.

We next set out to determine the molecular basis for the differential macrophage response to the LVS isolates. A blue to gray colony morphology variation has been described in LVS, where the gray variants are more proficient at activating host macrophages [157,162]. These blue/gray variants can be distinguished by immunoreactivity of their LPS because the blue strains react with antibodies against *F. tularensis* LPS and the gray strains react with antibodies recognizing *F. novicida* LPS [157,162]. Following proteinase K treatment of whole cell lysates from LVS and ACV, we compared the antibody reactivity of the remaining material to *F. novicida* LPS. An immunoblot of LPS from both LVS and ACV showed that both isolates reacted with the anti-*F. tularensis* LPS antibody and neither bound the anti-*F. novicida* LPS antibody (Figure 10). In contrast, the LPS isolated from *F. novicida* reacted strongly with the anti-*F. novicida* LPS antibody. Based on the molecular definition of Cowley et al. [157], our results indicate that both the LVS and ACV isolates exhibit the LVS blue phenotype.

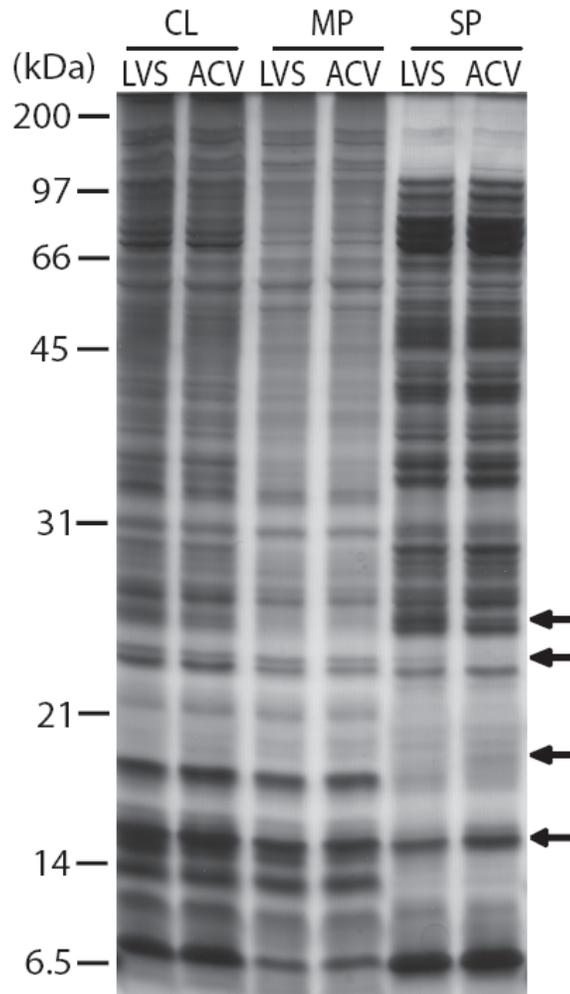


**Figure 10: Immunoreactivity of LPS from LVS and ACV.**

Proteinase K treated whole cell lysates from LVS and ACV were separated on a 4-12% Bis-Tris gradient gel by SDS-PAGE and transferred to PVDF membrane. After blocking, membranes were probed with anti- *F. tularensis* LVS or anti- *F. novicida* LVS monoclonal antibodies followed by anti-mouse IgG conjugated to Alexa Fluor 488 for detection. LPS from both LVS isolates reacted with anti-*F. tularensis* LPS monoclonal antibody (right) and anti-*F. novicida* LPS monoclonal antibody (left).

Since the immunoreactivity of the LPS was similar in the two isolates, we investigated the protein content of LVS and ACV to determine why they elicited different macrophage cytokine responses. As a first test, whole cell lysates, membrane proteins, or soluble proteins from each isolate were separated on single dimension acrylamide gels and silver stained to characterize bacterial proteins (Figure 11). The protein content in whole cell lysates and membrane fractions of LVS and ACV were indistinguishable using this technique. The soluble

fractions of LVS and ACV, however, demonstrated a number of differences observed by this method (Figure 11, arrows).

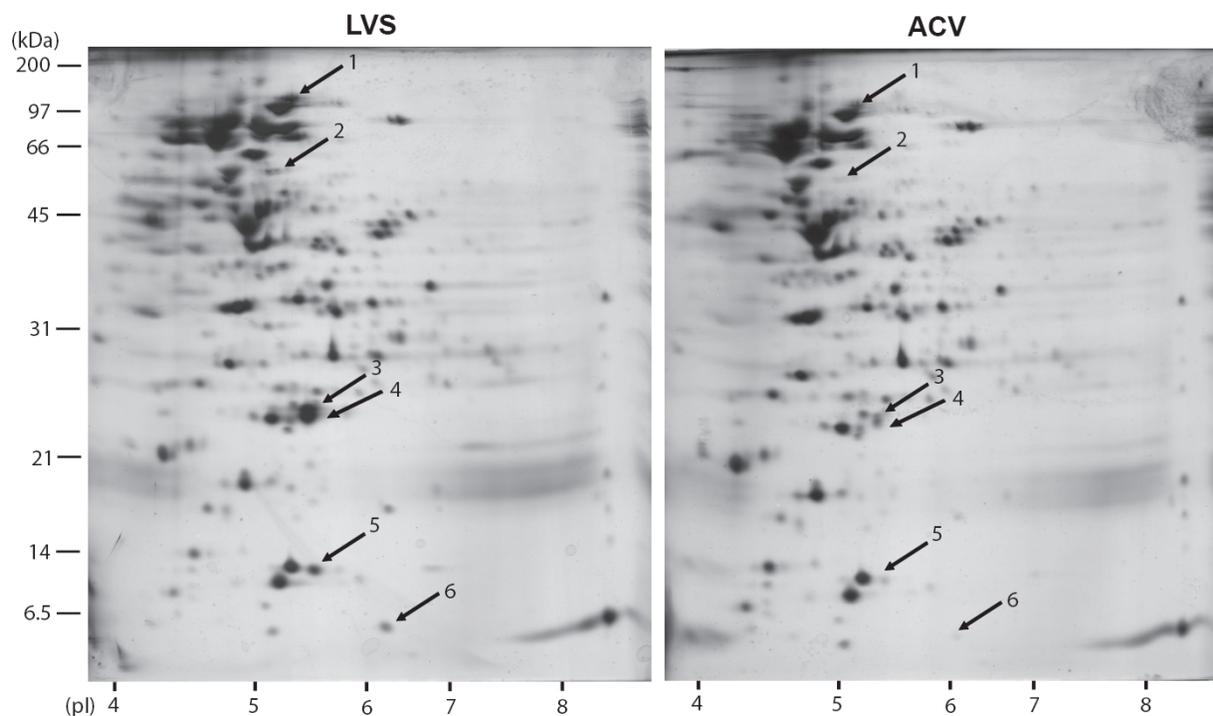


**Figure 11: Differences in protein expression between LVS and ACV.**

Whole cell lysates or subcellular fractions of LVS and ACV were separated on a one-dimensional SDS-PAGE gel (12.5% acrylamide). The gel was stained with silver reagents. Cellular fractions are labeled: CL = cell lysate, MP = membrane proteins, and SP = soluble proteins. Apparent differences of protein content in the soluble fraction are indicated with arrows.

We pursued the differences in the soluble protein fractions of LVS and ACV using two-dimensional gel electrophoresis and silver staining. Using this approach, we found the majority

of proteins to be similar in abundance between LVS and ACV when equal amounts of protein were loaded on the gel. However, six proteins were identified that were qualitatively greater in LVS than in ACV (Figure 12). The spots containing these proteins were isolated and identified by MALDI-TOF (Table 3). One of the proteins identified was intracellular growth locus C (IglC), a known *F. tularensis* virulence factor [54,55,64,71,73,74]. Also included were aconitase, which is part of the TCA cycle and has been associated with *Staphylococcus aureus* virulence [163], and a homolog of a  $\sigma^{54}$  modulation protein (YhbH), which has known functions in sporulation [164], biofilm formation [165], and quorum sensing [166]. Other identified proteins included fumerate hydratase, a protein involved in the TCA cycle, and a heat shock protein (Hsp20). Proteins that appeared more abundant in ACV (Figure 11) were not identified on the two-dimensional gels (Figure 12). This was most likely related to differences in protein solubilization between the techniques; SDS was used in one-dimensional SDS-PAGE. In addition, a narrower portion of the pH range (pH 4 to 8.5) was resolved in the isoelectric focusing step of the two-dimensional gel protocol than the total proteins contained in one-dimensional gels. Nevertheless, qualitative differences in protein content were observed using the two-dimensional electrophoresis technique.



**Figure 12: Identification of differences in the soluble protein fractions of LVS and ACV by two-dimensional gel electrophoresis.**

Soluble protein fractions of LVS and ACV were separated in first dimension by isoelectric focusing and by size in second dimension on a 12.5% acrylamide gel. Silver-stained gels contained multiple differentially-expressed proteins between LVS and ACV. Specific proteins are numbered and are identified in Table 3. Only those spots reproducibly different in two different protein isolations and on five repeated gels are labeled. Positions of molecular mass standards (kDa) and pI range are indicated on the left and bottom, respectively.

**Table 3: MALDI-TOF identification of proteins present in LVS but reduced in ACV**

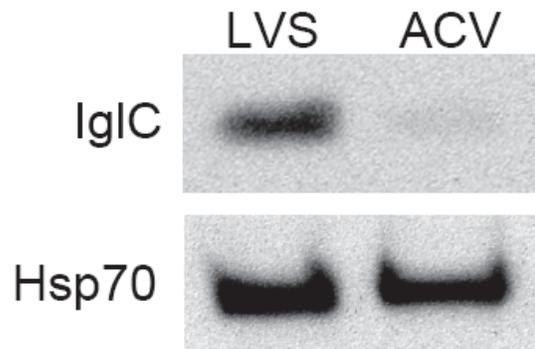
Spot	Identification (locus designation)	% Cov. <sup>a</sup>	MP <sup>b</sup>	Theoretical <sup>c</sup>		Observed	
				pI	mass (Da)	pI	mass (Da)
1	Aconitate hydratase, Aconitase, <i>acnA</i> homolog (FTT0087)	37	42	5.4	102,615	5.4	102,450
2	Fumerate hydratase, <i>fumA</i> homolog (FTT1600c)	45	26	5.2	54,974	5.2	58,340
3	Intracellular growth locus subunit C, <i>iglC</i> (FTT1712c or FTT1357c)	37	9	6.3	22,433	5.6	22,000
4	Intracellular growth locus subunit C, <i>iglC</i> (FTT1712c or FTT1357c)	40	11	6.3	22,433	5.6	21,590
5	Heat shock protein, <i>hsp20</i> homolog (FTT1794)	33	5	5.7	16,712	5.7	14,410
6	Sigma-54 modulation protein, <i>yhbH</i> homolog (FTT1281c)	46	8	6.3	11,182	6.3	12,660

<sup>a</sup> Percent coverage of the identified protein

<sup>b</sup> Number of matching peptides

<sup>c</sup> Obtained from the TIGR website and does not include post translational modifications.

We next sought to confirm the results obtained by two-dimensional gels with another technique. To do this, we measured IglC levels by immunoblot to quantify the difference between LVS and ACV whole cell lysates. IglC levels were 15-fold higher in LVS than in ACV (Figure 13), confirming the qualitative results observed with silver stain. We have previously observed that Hsp70 levels of LVS and ACV were comparable on two-dimensional gels and immunoblots derived from two-dimensional gels, and used this to confirm protein loads. The membrane was re-probed with anti-Hsp70 polyclonal antibody showing similar levels of Hsp70 in the 1 $\mu$ g of whole cell lysates that were loaded in each lane (1.2-fold difference) (Figure 13).



**Figure 13: Quantification of IglC levels by immunoblot.**

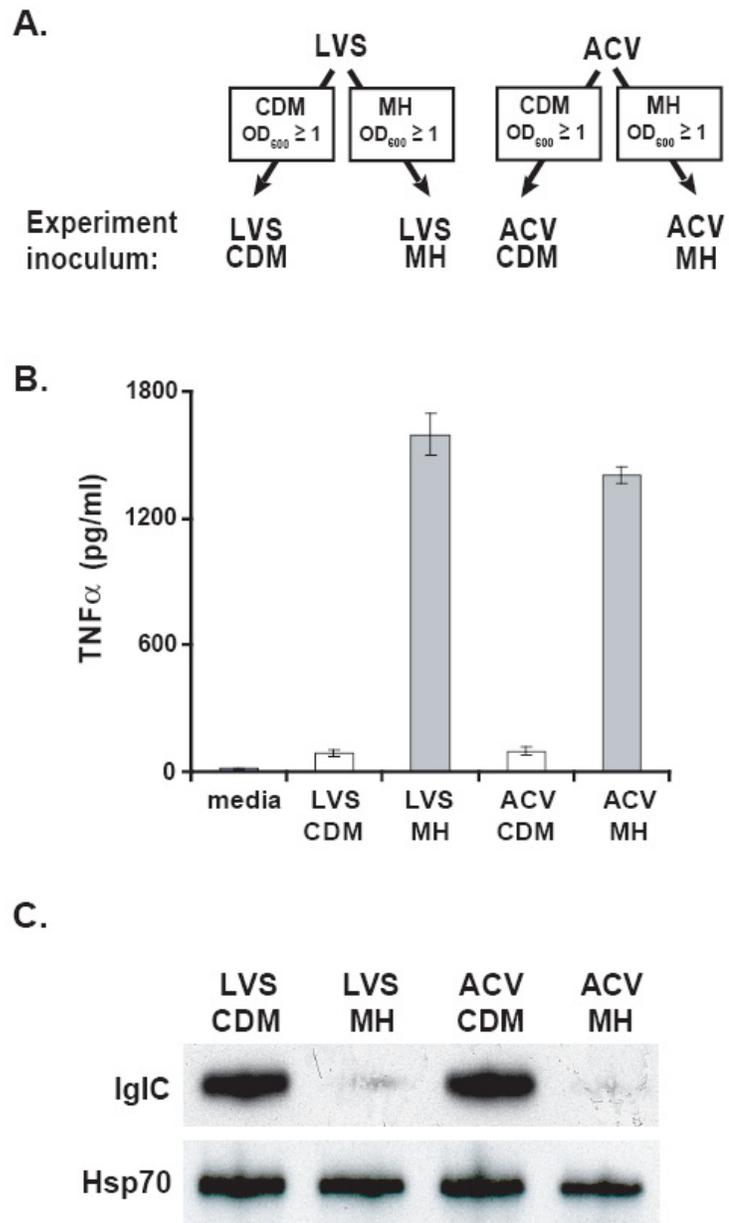
Whole cell lysates from LVS and ACV were separated by SDS-PAGE (12% acrylamide) and transferred to PVDF membrane. After blocking, the membrane was probed for IglC expression (top), using monoclonal mouse anti-IglC and anti-mouse IgG conjugated to HRP, and then re-probed with polyclonal anti-Hsp70 to assess protein loads (bottom). Levels of IglC and Hsp70 expression were quantified using densitometry. IglC expression was 15-fold higher in LVS samples, while Hsp70 controls exhibited less than 1.5-fold differences.

We next assayed the expression of several genes to determine if RNA levels correlated with the protein levels observed on the two-dimensional gels (Figure 11). We selected *acnA*, *iglC*, and *yhbH* for this test as each has been associated with bacterial virulence [55,64,163-166]. The abundance of these genes' transcripts was measured by quantitative PCR using 16S rRNA as an internal reference. Surprisingly, the expression of the *iglC*, *acnA*, and *yhbH* differed less than two-fold between LVS and ACV (data not shown). The presence of transcripts for these genes argues against the hypothesis that genomic deletions in ACV explain loss of IglC, AcnA, and YhbH. Rather, the gene expression data suggest the amounts of these three proteins were controlled at a step after transcription.

#### 2.4.4 Reversion of ACV to the LVS phenotype.

We next sought to determine if ACV could revert back to the LVS phenotype. To investigate this, we tested different culture conditions and media, including a chemically defined media (CDM) originally described by Chamberlain [158]. For these experiments, LVS and ACV were cultivated in CDM or MH. The procedure for generating these experimental inocula is illustrated in figure 14A. As previously observed (Figure 7), growing LVS to high density ( $OD_{600} \geq 1.0$ ) in MH broth elicited the ACV phenotype and high levels of TNF $\alpha$  production from macrophages (“LVS MH”, Figure 14B). When ACV was used to inoculate MH broth, high levels of cytokine were observed again (“ACV MH”, Figure 14B). When LVS was grown to a high density in CDM ( $OD_{600} \geq 1.0$ ), it retained its phenotype and induced little TNF $\alpha$  (“LVS CDM”, Figure 14B). More importantly, ACV grown in CDM behaved like LVS eliciting little TNF $\alpha$  (“ACV CDM”, Figure 14B). These results suggest that ACV had reverted to LVS following cultivation in CDM.

We then tested whether the ACV reversion correlated with a change in IglC after culture in the CDM. For this, we utilized the same bacterial cultures used to infect macrophages (Figure 14C). Growth to a high density in MH resulted in low levels of IglC, irrespective of the source of the initial inoculum (“LVS MH”, “ACV MH”; Figure 14C). This result is similar to our previous data showing low IglC expression in ACV (Figure 12, 13). In contrast, cultures grown in CDM had substantially higher levels of IglC, once again irrespective of which strain was used to inoculate the culture (“LVS CDM”, “ACV CDM”; Figure 14C). These data indicate an ability of *F. tularensis* to modulate expression of its virulence factors based on its environment.



**Figure 14: ACV reversion to the LVS phenotype.**

(A.) Schematic representation of strains and growth conditions used in reversion experiments. LVS and ACV were grown in CDM or MH to a high density ( $A_{600} \geq 1.0$ ). LVS grown in CDM or MH are designated “LVS CDM” and “LVS MH.” Similarly, ACV grown in CDM or MH are designated “ACV CDM” and “ACV MH.” Bacteria were harvested from these overnight cultures and were used for the experiments described in B and C.

(B.) ACV induced low levels of TNF $\alpha$  following growth in CDM. Macrophages were incubated with media only (“media”) or in the presence of the four bacterial cultures at an MOI of 10 for 24 hours. TNF $\alpha$  levels were measured by ELISA. Data are mean  $\pm$  SD of triplicate wells within one experiment. Data are representative of 3 individual experiments with macrophages derived from different donors.

(C.) IglC protein expression increased following growth of ACV in CDM. Aliquots of the cultures used in part B were lysed using LDS loading buffer (Invitrogen). After SDS-PAGE of whole cell lysates and transfer, the PVDF membrane was probed for IglC expression (top) and then re-probed for Hsp70 levels to assess protein loads (bottom). IglC levels were 30 and 60 fold greater in LVS and ACV, respectively, following growth in CDM. Hsp70 loading controls exhibited less than 1.5 fold differences.

## 2.5 DISCUSSION:

Although *F. tularensis* can cause a life-threatening disease in humans, current knowledge of the host-pathogen interaction is incomplete, particularly in human cells. In the course of our studies, we have isolated a variant of *F. tularensis* LVS based on colony morphology. Comparing the response of human macrophages to both the parent isolate (LVS) and the variant (ACV), we found they induced different amounts of cytokines. ACV consistently induced higher amounts of each proinflammatory cytokine tested. Both of our isolates are classified as “blue” based on LPS immunoreactivity [157], so we sought other explanations for the differences in macrophage activation. By comparing the proteomes of these two closely related isolates, we found differences in protein levels for the well-characterized *F. tularensis* virulence factor IglC, as well as proteins associated with virulence in other organisms. These differences likely contribute to the macrophage responses we observed (Figure 7).

Our parent isolate exhibits a phenotype comparable to other published reports, while ACV appears unique. For example, Bosio and Dow showed that LVS does not induce detectable levels of TNF $\alpha$  from murine pulmonary macrophages or dendritic cells [167]. Our results are also consistent with other observations using human macrophages, where LVS induces significantly less TNF $\alpha$  than that elicited by *E. coli* or *E. coli* LPS [156]. ACV failed to recapitulate these typical LVS phenotypes. ACV induced high levels of TNF $\alpha$  and other pro-inflammatory cytokines. The fact that our isolates were stable on sub-culturing on both chocolate agar and MH broth (grown to low density,  $A_{600} < 0.2$ ) provided a useful system to contrast the protein expression profiles of LVS and ACV.

Examination of soluble protein fractions by two-dimensional gel analysis permitted us to identify six protein spots that were expressed at much higher levels in LVS (Figure 12). Previous *Francisella* proteomics studies have catalogued LVS protein profiles [168,169], immune reactive proteins [170], or stress response proteins [130]. A proteomic comparison of three *Francisella* strains has been performed, identifying many protein differences [171]. However, most of these were mass and charge variants of proteins present in all three strains [171]. The relatedness of our isolates facilitated the identification of protein differences beyond size and charge variations. Importantly, we were able to correlate altered protein content (Figure 12) with physiologic responses by host cells (Figure 7, 14).

The differences between LVS and ACV proteomes included proteins with roles in bacterial virulence. The most notable of these is intracellular growth locus C (IglC), a virulence factor known to play a role in *F. tularensis* and *F. novicida* intramacrophage survival [9,61,64,74]. Increased expression of IglC has also been demonstrated following growth in macrophages and exposure to hydrogen peroxide [77], suggesting a role for this protein in the stress response of the bacteria. Strains deficient in IglC are unable to grow within macrophages or escape from phagosomes [9,55,64]. LVS also inhibits TLR signaling in macrophages and IglC mutants do not manifest this phenotype [127]. ACV exhibits a phenotype similar to an IglC mutant, and is unable to inhibit TLR signaling (Figure 9). It is likely that the lower abundance of IglC in ACV (Figure 12) contributes to the heightened macrophage response induced by this strain.

Other proteins we identified have been associated with virulence in other systems. Aconitase, a TCA cycle protein that catalyzes the conversion of citrate to isocitrate, has been implicated in *S. aureus* virulence. Aconitase gene deletions in *S. aureus* reduce virulence factor

expression and slow onset of subcutaneous infections in mice [163]. In *Pseudomonas aeruginosa*, aconitase contributes to synthesis of exotoxin A [172]. Although *F. tularensis* aconitase protein levels are lower in mouse spleen four days after inoculation than in broth culture [173], the role of this enzyme in other stages of *Francisella* infection has not been investigated.

We also found higher protein levels of YhbH, a member of the  $\sigma^{54}$  modulation protein family, in LVS. YhbH has been implicated in virulence of *Bacillus cereus* for its roles in sporulation [164] and biofilm formation [165]. In *E. coli*, YhbH is known to play a role in quorum sensing [166] and stabilizing ribosomes [174]. Biofilms and quorum sensing have important roles in virulence in several microbiological systems [175,176], yet these properties have not yet been described in *F. tularensis*. The ability to stabilize ribosomes could lead to increased translation of virulence factors, like IglC, or other proteins without necessarily increasing transcription. Improved expression of virulence factors and ability to translate proteins could enhance *F. tularensis* survival in hostile conditions.

Although multiple differences were observed between LVS and ACV at the protein level, mRNA levels were similar for all three of the genes tested. This indicates that loss of genetic material is not the mechanism of the variation observed and suggests post-transcriptional events, such as differential protein stability and selective protein degradation, may regulate the expression of proteins like IglC. Importantly, regulation of translation has been implicated in the control of genes associated with virulence in several other organisms such as *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Burkholderia cenocepacia* [177-180].

Our results provide a molecular explanation to other publications regarding LVS, virulence, and host responses. CDM is known to enhance the virulence of LVS vaccine stocks

[181]. Cherwonogradzky and colleagues showed growth of LVS in CDM increased capsule production and virulence in mice by approximately 1000-fold [181]. Our data suggest levels of IglC, and possibly other virulence determinants, increased by growth in CDM contribute to these observations. Loegering *et. al.* recently demonstrated LVS grown in a murine macrophage-like cell line, RAW 264.7, induce lower amounts of proinflammatory cytokines than bacteria grown in broth cultures [132]. Our results suggest the LVS grown in MH for their studies had shifted to the ACV variant, inducing cytokines because of a low level of IglC. Passage of their bacteria through macrophages reconstituted the LVS phenotype with low cytokine induction, most likely because growth of LVS in macrophages increases IglC expression [77].

Our results are the first to demonstrate modulation, up and down, of *F. tularensis* virulence factor expression in response to environmental cues. ACV reversion to LVS was observed following overnight growth of bacteria in CDM (Figure 14). This phenotype switch was associated with reconstitution of IglC levels. The signal(s) that increase virulence factors in LVS and down-regulate macrophage responses will be the focus of future studies. It is of note that MH and CDM have substantial differences, including amounts of iron (100-fold difference), phosphate (4-fold) and potassium (10-fold).

We have shown the two isolates of *F. tularensis* LVS induce different amounts of proinflammatory cytokines from human macrophages, with the ACV inducing higher levels of all cytokines tested. Proteomic analysis of these isolates identified both known (IglC) and candidate (AcnA, YhbH) *F. tularensis* virulence factors. The ability of ACV to revert to an LVS phenotype when grown in CDM provides evidence of virulence factor modulation based on environment, though the exact nature of the signals/receptors leading to these changes remains to be determined. Further study of the RNA and protein differences between LVS and ACV creates

a unique opportunity to discover candidate *Francisella* virulence factors whose expression correlate with known virulence factors like IglC and to investigate virulence factor regulation, ultimately increasing our understanding of *F. tularensis* pathogenesis.

### **3.0 CHAPTER 2: DETECTION OF THE HOST CYTOSOLIC ENVIRONMENT IS ESSENTIAL FOR *FRANCISELLA* SURVIVAL**

#### **3.1 ABSTRACT:**

Tularemia is caused by the Category A biodefense agent, *Francisella tularensis*. This bacterium is associated with diverse environments and a plethora of arthropod and mammalian hosts. How *F. tularensis* adapts to these different conditions, particularly the eukaryotic intracellular environment in which it replicates, is poorly understood. Here we demonstrate the polyamine spermine, an abundant intracellular molecule produced exclusively by eukaryotic cells, is an environmental signal that induces a more virulent phenotype in *F. tularensis*. Genome-wide analysis showed *F. tularensis* LVS undergoes considerable changes in gene expression in response to spermine, including known and candidate virulence factors. Unexpectedly, analysis of gene expression levels showed that multiple members of two classes of *Francisella* IS elements, *isftu1* and *isftu2*, and the genes adjacent to these elements were induced by spermine. Spermine was sufficient to activate transcription of these IS elements and of nearby genes in broth culture and in macrophages. Disruption of an ABC transport system, annotated for polyamine uptake, eliminated the ability of *F. tularensis* LVS to respond to spermine. The mutant bacteria elicited greater cytokine production from macrophages than wild-type bacteria, were unresponsive to extracellular spermine, were attenuated for growth in human macrophages,

and were less virulent in an *in vivo* model of infection. Our results demonstrate for the first time spermine recognition is a novel, essential mechanism for *F. tularensis* adaptation to a eukaryotic host environment and intracellular survival. Other bacteria, including intracellular pathogens, have similar polyamine ABC transporters, implicating this system in other host-pathogen interactions.

### 3.2 INTRODUCTION:

*Francisella tularensis* is a formidable pathogen, leading to its designation as a category A Biodefense agent [2]. Among its many characteristics is the ability to replicate in macrophages [1]. Upon infection of these cells, *F. tularensis* encounters molecular cues that alert this pathogen to its new environment. The ability of *F. tularensis* to detect and respond to environmental signals contributes to this organism's success as a pathogen. For example, iron restriction and oxidative stress, conditions encountered within a mammalian host, induce the expression of genes critical for the establishment of a successful infection [12,77,182]. Understanding the mechanisms by which *F. tularensis* adapts to its environment may provide insight into the pathogenicity of this organism.

An effective immune response to *F. tularensis* requires innate immunity. Proinflammatory cytokines, including TNF $\alpha$  and IFN $\gamma$  play a major role in controlling *Francisella* infections [104]. Both T-cell deficient and *scid* mice exhibit heightened sensitivity to LVS when depleted of these cytokines [105]. These mice are killed by a sublethal dose of LVS within one week post infection, indicating that these cytokines play a vital role in early stages of infection [105]. Despite the importance of innate immunity, *Francisella* actually inhibits the production of proinflammatory cytokines by macrophages, providing the bacterium with a more hospitable environment [108,127,128].

We have previously shown that the growth conditions of *F. tularensis* live vaccine strain (LVS) affect both the production of a known virulence factor and the ability of the bacterium to inhibit macrophage cytokine production [108]. Growth of LVS in a chemically defined medium

(CDM) [158] elicits an inhibitory phenotype, while bacteria grown in Mueller Hinton (MH) broth induce cytokine responses from macrophages. The chemical signals in CDM and MH responsible for these bacterial phenotypes are poorly understood.

Polyamines, including putrescine, spermidine, and spermine, are cationic compounds found in all living organisms. These molecules reach millimolar levels in both prokaryotic and eukaryotic cells, but spermine is only produced by eukaryotes [135]. Polyamines are associated with DNA synthesis, transcription, translation, and enzyme activity [133,183]. These molecules are known to induce global gene regulation in *Escherichia coli* leading to optimal growth [144]. Polyamines are essential for biofilm formation in both *Yersinia pestis* and *Vibrio cholera* [139,152] and contribute to *Streptococcus pneumoniae* virulence through an unknown mechanism [151].

In this work, we have investigated the molecular mechanisms responsible for differential cytokine induction by *F. tularensis* [108]. We have identified an environmental signal, spermine, that alters LVS behavior, characterized the bacterial response to this signal, and identified the receptor system that is required. These results define a novel mechanism of virulence gene regulation in this Category A Biodefense Agent and have implications for other pathogens.

### 3.3 MATERIALS AND METHODS:

#### Francisella strains and cultivation:

*F. tularensis* LVS was kindly provided by Dr. Karen Elkins (U.S. Food and Drug Administration). For macrophage infections, LVS was grown on chocolate II agar for 1-3 days at 37°C and 5% CO<sub>2</sub>. Mueller-Hinton broth supplemented with 0.1% glucose, 0.025% ferric pyrophosphate (Sigma), and Isovitalex (Becton Dickinson) was used to grow overnight cultures of LVS. For experiments including spermine, cultures were supplemented with 200 µM spermine (Sigma), an amount equal to that used in a chemically defined medium for cultivation of *F. tularensis*, unless otherwise indicated [158]. Following overnight growth, bacteria were centrifuged, washed twice in DMEM and resuspended in infection media (DMEM supplemented with 1% human serum, 25mM HEPES (Invitrogen), and 1X Glutamax (Invitrogen)). Bacteria were then diluted to an appropriate multiplicity of infection (MOI) prior to infection. All MOI were confirmed by plating for viable cfu.

#### Macrophage culture and infection:

Macrophage cultures were performed as described previously [108]. Unless otherwise indicated, macrophages were co-cultured with bacteria at a MOI of approximately 10 for 24 hours before supernatants were collected for analysis of cytokine levels. For bacterial intramacrophage growth curves and fluorescence microscopy, macrophages were infected at an MOI of 100 for one hour at 37°C. The high MOI was used to increase the percent macrophages (> 80%) infected following the short incubation. Macrophages were then treated with gentamicin

(20µg/ml) and washed three times with HBSS, to remove extracellular bacteria. At the time points indicated, the macrophages were lysed using 0.02% SDS and serial dilutions of bacteria were plated to enumerate cfu. All use of human-derived cells was approved by the University of Pittsburgh Institutional Review Board.

For RAW264.7 infections, the cell line was grown to about 80% confluency in 75cm<sup>2</sup> flasks prior to infection (approximately 2x10<sup>7</sup> cells/flask). LVS was grown in MH broth overnight, washed, and resuspended in macrophage infection media (DMEM with 1% serum). A portion of the washed bacteria were harvested at this point for RNA to compare to LVS RNA recovered from the RAW264.7 cells. RAW264.7 cells were infected with LVS at an MOI of 500 for 2 hours to establish a high infection rate. After this 2 hour incubation, the cultures were then treated with gentamicin (20µg/ml) and washed three times with HBSS to remove extracellular bacteria. Bacteria were allowed to replicate intracellularly for 48 hours. Following the 48 hour incubation, extracellular bacteria were washed away using PBS and RNA was isolated from the flask.

#### ELISA analysis:

Macrophage supernatants, harvested 24 hours following the introduction of bacteria, were subjected to ELISA analysis to measure TNFα production using a matched antibody pair (R&D Systems). Following the addition of TMB substrate solution (Dako) and measurement of optical density using a Molecular Dynamics M2 plate reader, cytokine levels were calculated from a standard curve. The limit of detection for the TNFα ELISA was 15 pg/ml.

#### RNA harvest and analysis:

RNA was harvested from broth cultures by adding 5ml of a 16 hour culture directly to 15 ml TriReagent LS (Molecular Research Center). For RAW264.7 cultures, 15 ml of TriReagent was added to the tissue culture flask after washing. RNA extraction continued by adding a 1/5 volume of chloroform, then the aqueous phase was separated by centrifugation in a Phase Lock Heavy tube (Eppendorf). RNA was precipitated with isopropanol, followed by centrifugation. Pellets were washed with 80% ethanol and resuspended in nuclease-free water. Fifty  $\mu$ g of the RNA-containing mixture was treated with DNase (Turbo DNA-*free*, Ambion), followed by ammonium acetate precipitation. RNA quantity was measured by spectrophotometry and quality with an Agilent Bioanalyzer.

#### Microarray design:

Custom *Francisella* microarrays were designed using Agilent's eArray framework. All ORFs, including pseudogenes, were included for *F. tularensis* spp. *tularensis* (SchuS4), *F. tularensis* spp. *holarctica* LVS, *F. tularensis* spp. *holarctica* OSU18, *F. novicida* U112 and the *Francisella* plasmids, pOM1, and pFNL10. Each gene from SchuS4, LVS, and OSU18 was spotted in duplicate on the array, while the others were included as single copies.

#### Microarray target preparation and hybridization:

Synthesis of labeled cDNA target was performed using 10  $\mu$ g of total RNA, 0.5  $\mu$ g random hexamers (Invitrogen), and MMLV (Agilent). cDNA target was labeled with Alexa Fluor 555 conjugated dUTP according to manufacturer's protocol (AF555-aha-dUTP; Invitrogen). Reactions were incubated at 40°C for 3 hours. Following cDNA synthesis, remaining RNA was hydrolyzed with 10 $\mu$ l 1N NaOH and 10 $\mu$ l 0.5M EDTA. pH was then neutralized with 10  $\mu$ l 1M

HCl and the labeled cDNA was precipitated using ammonium acetate/isopropanol and washed with 80% ethanol. 0.5µg of labeled cDNA was hybridized to custom Agilent 8 x 15K microarrays according to the manufacturer's protocol and incubated at 60°C for 18 hours in a rotary oven. Following hybridization, arrays were washed with Agilent wash buffers before being scanned on an Agilent microarray scanner.

#### Microarray data analysis:

Analysis of the microarray data was carried out using Gene Expression Data Analyzer (GEDA; <http://bioinformatics.upmc.edu/GE2/GEDA.html>) [184]. Briefly, this online software package was used for median normalization within an array followed by log<sub>2</sub> transformation. Data were grouped into categories, with or without spermine, and subjected to the J5 statistical analysis, which is designed for data sets with limited numbers of replicates and reduces the chances of false positives [184]. Genes were considered statistically significant if they had a J5 score greater than 2. Tables 5 and 6 report both the J5 value and fold change for genes that changed significantly. Genes meeting these requirements were then input into GenePattern [185] and data are presented as a hierarchical clustering (Pearson correlation) of log<sub>2</sub> transformed normalized intensity values for these genes.

#### Quantitative PCR:

cDNA synthesis was performed using Superscript III (Invitrogen) and 1µg of total RNA. Real time reactions were performed with a 1:5000 final dilution of template cDNA. Primer sets were designed for genes indicated using Primer 3 [186] and reactions were carried out on an BioRad IQ5 real time machine using SYBR Green (BioRad). The bacterial 50S ribosomal protein L18

gene (FTL\_0252) was used as the internal reference, as it was observed to have no change in expression on microarray data (data not shown). The Q-PCR data are presented as  $\log_2$  transformed fold change values (MH+spermine/MH alone).

#### Construction of *potF* mutant:

A single cross over strategy [187] was utilized to disrupt *potF* from the *F. tularensis* LVS genome. To do this, the central 900 bp of the gene were PCR amplified using the *potF* disruption primers (table 4) and cloned into pGEM-T (Promega). The Sall fragment of this vector containing *potF* sequence was subcloned into pPV [74] digested with this same enzyme producing pPV-potF. A homologous recombination between the LVS chromosomal *potF* and the pPV-potF truncated copy (the central 900 bp of this gene) would disrupt coding sequence for the 30 C-terminal amino acids of PotF (data not shown). This region includes conserved sequence essential for polyamine binding of the *E. coli* PotF homolog [188]. pPV-potF was mobilized to LVS using triparental conjugation [189]. Mating mixes were composed of LVS ( $10^9$  cfu), *E. coli* DH5 $\alpha$  / pPV-potF ( $10^7$  cfu), and *E. coli* HB101 / pRK2013 ( $10^7$  cfu). Similarly to the previously described two-strain *Francisella* conjugation technique [74], the triparental mating mixture was plated on LB agar plates, and incubated at room temperature for 18 hours. Cells were resuspended in phosphate buffered saline and dispensed onto chocolate II agar plates containing polymyxin (100 $\mu$ g/ml) and chloramphenicol (2.5 $\mu$ g/ml). Chloramphenicol resistant colonies were screened by PCR using primers specific for *potF* and pPV to confirm integration (data not shown). The LVS *potF* disruption mutant (*potF*::pPV-potF) is referred to as LVS *potF*-I. All strains used in this chapter are listed in Table 4.

#### Construction of *isftu* reporter constructs:

Constructs were designed using a red fluorescent protein, tdTomato [190], as a reporter to measure gene expression. *Isftu1* and *isftu2* sequences were amplified from the LVS genome along with the upstream 30bp and 300bp, respectively, using PCR. Upstream sequences that were included were chosen based on sequence homology among ten representative members of each transposase. *Isftu* sequences were ligated into pRSETB-tdtomato using either XbaI (*isftu1*) or NheI (*isftu2*) sites and BamHI sites. The *Isftu*-tdtomato fragment was then subcloned into pFNLTP8 using either XbaI (*isftu1*) or NheI (*isftu2*) sites and EcoRI sites. Plasmids were electroporated into *F. tularensis* as previously reported [191]. Briefly, *F. tularensis* LVS cultures (approximately 50 ml) supplemented with 0.1% cysteine (TSB-C) were grown to an OD<sub>600</sub> of approximately 0.4, washed three times with 500mM sucrose, and resuspended in 4 ml of 500mM sucrose. For electroporation, 1 µl (approximately 1 µg) of plasmid DNA was mixed with 50 µl of electrocompetent cells and pulsed in a 0.2 cm gap cuvette at 2.5 kV, 150 Ω, and 25 µF. Bacteria were immediately resuspended in 1 ml of trypticase soy broth supplemented with 0.1% cystine (TSB-C) and grown at 37 °C for 4 hours before selection on chocolate II agar plates supplemented with kanamycin (10µg/ml). All vectors and the primers used to create them are listed in Table 4. Individual colony transformants were then patched onto chocolate agar plates containing kanamycin. These patches were used to inoculate overnight cultures in MH broth either with or without supplemental spermine. Cultures were diluted two fold before measuring fluorescence and OD<sub>600</sub> using a multi-label plate reader (Molecular Devices).

#### Chicken embryo infections:

Chicken embryos were infected with *F. tularensis* LVS and *potF-1* as previously described [75,192]. Specific pathogen free, fertile, Whiteleghorn chicken eggs were purchased from Charles River Laboratories, North Franklin, CT, USA. Eggs were incubated at 37 °C with humidity and gentle rocking for 7 days prior to infection. Following the initial 7 day incubation period, eggs were candled to check embryo viability, and those without a viable embryo were discarded. To infect the embryos, the egg shell surface was sterilized with 70% ethanol. A small hole was formed at the air sac end of the egg to expose the egg shell membrane. After removing the egg shell membrane, 100µl of bacteria suspended in PBS were injected beneath the chorioallantoic membrane. Clear tape was used to seal the hole formed in the shell and eggs were incubated as described above. Eggs were candled daily to check viability for 6 days. As previously reported, embryos that died within 24 h of the infection were presumed to have suffered lethal trauma during inoculation, and were removed from the experiment. Differences in survival in chicken embryo infections were analyzed with the log rank test in GraphPad Prism 5.

**Table 4: Plasmids, strains, and primers used in this chapter.**

<b>Plasmid</b>	<b>Description</b>	<b>Ref.</b>
pRK2013	Helper plasmid for triparental mating, Km <sup>R</sup>	[189]
pPV	<i>Francisella</i> suicide vector, oriT, Cm <sup>R</sup>	[74]
pPV-potF	pPV + 900bp central <i>potF</i> sequence	this study
pRSET <sub>B</sub> -tdtomato	Source of <i>tdtomato</i> , Km <sup>R</sup>	[190]
pFNLTP8	<i>Francisella</i> shuttle vector, Km <sup>R</sup>	[193]
pF8-tdtomato	pFNLTP8 + promoterless <i>tdtomato</i>	this study
pF8-isftu1-tdtomato	pFNLTP8 + <i>isftu1-tdtomato</i> reporter sequence	this study
pF8-isftu2-tdtomato	pFNLTP8 + <i>isftu2-tdtomato</i> reporter sequence	this study

<b>Strain</b>	<b>Description</b>	<b>Ref.</b>
LVS	<i>F. tularensis</i> live vaccine strain	K. Elkins
<i>potF-1</i>	LVS with FTL_1582 ( <i>potF</i> ) disrupted by pPV-potF	this study
<i>potF-R</i>	Revertant of <i>potF-1</i> with wild-type FTL_1582	this study

<b>Primers</b>	
<i>potF</i> disruption	FWD: 5' - CCAGT <u>CGAC</u> GTAGGTCTGATATTAGTATCATCAAAGATATCA REV: 5' - CCAGT <u>CGAC</u> AGACTATATATCTCCATATATAGTACCATGTTTTT
<i>Isftu1</i>	FWD: 5' - CAAT <u>CTAGA</u> AATATAGTTAATCCGAAAGATATTTGTAGAAAAAG REV: 5' - CAAGGATCCTTAATCCGAGAGTATTTTGTAAACACA
<i>Isftu2</i>	FWD: 5' - CAAGCTAGCTAGGTTCTGTGCACAAAAAACTTAA REV: 5' - CAAGGATCCTCAGGTTATCATTTGGTAAAGGTGTAA

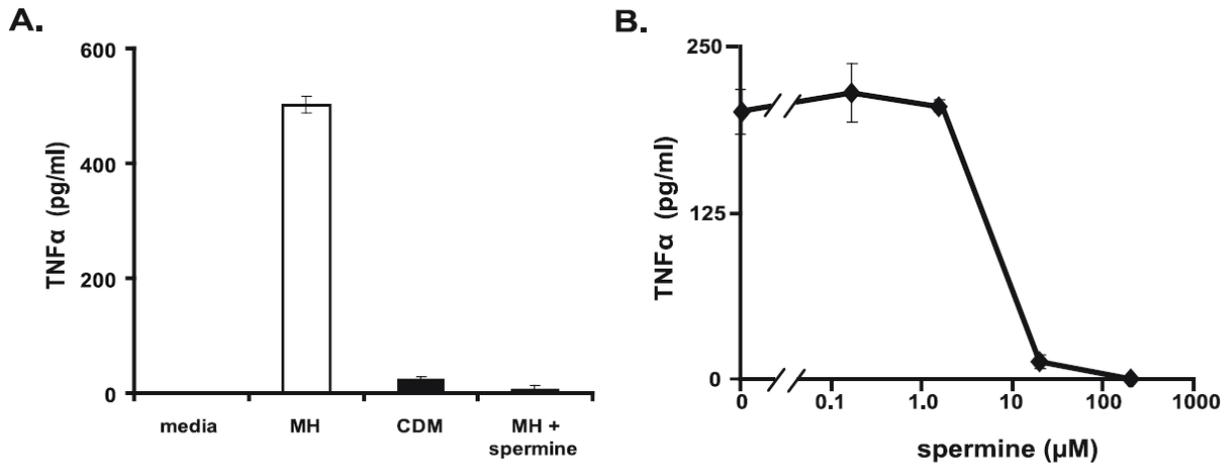
## 3.4 RESULTS:

### 3.4.1 Human macrophage response to *Francisella* following cultivation in the presence of spermine:

Previously, we have shown that *F. tularensis* LVS induces different macrophage responses depending on the media used to cultivate the bacteria [108]. Growth to high density in MH broth induces a bacterial phenotype that stimulates macrophages [108]. Alternately, high density growth in CDM reverts the variant to the parent LVS phenotype which actively inhibits cytokine production by macrophages [108]. One major difference between these two media is the presence of high levels of spermine in the CDM [158]. Since polyamines, such as spermine, have been shown to have significant effects on various processes in other bacteria [139,144,151,152], we sought to determine if the spermine component of CDM contributed to the LVS phenotype that inhibits macrophage activation. LVS was grown in CDM, MH broth, or MH broth supplemented with spermine at a concentration equal to CDM (200 $\mu$ M), and bacteria from these cultures were tested for their ability to stimulate human monocyte-derived macrophages. Macrophages infected with bacteria that had been grown in MH broth produced high levels of proinflammatory cytokines, while those grown in CDM induced substantially less (Figure 15A), consistent with our previous observations [108]. Macrophages infected with bacteria grown in MH broth supplemented with spermine produced significantly less TNF $\alpha$  compared to bacteria grown in MH alone (Figure 15A, (p<0.01)), a phenotype previously associated with wild-type LVS [108]. The reduced cytokine response to LVS grown in MH plus

spermine was not the result of altered bacterial growth state. Growth rates of LVS in MH or MH with supplemental spermine were identical in broth cultures and intracellularly following macrophage infection (data not shown).

We next sought to determine the range of spermine concentrations required for the phenotypic changes. Bacteria were grown in MH broth supplemented with decreasing amounts of spermine (200 $\mu$ M, 20 $\mu$ M, 2 $\mu$ M, 0.2 $\mu$ M, and 0 $\mu$ M) prior to infection of macrophages. Reduced cytokine production was observed in response to bacteria grown in concentrations of spermine as low as 20 $\mu$ M (Figure 15B). These data indicate that LVS responds to spermine at concentrations two orders of magnitude lower than the free spermine concentration in eukaryotic cells [135].



**Figure 15: Inhibition of macrophage cytokine response by *Francisella* grown in the presence of spermine.**

(A) TNF $\alpha$  production by human monocyte derived macrophages following exposure to *F. tularensis* LVS grown in indicated media at an MOI of 10 for 24 hours. Uninfected control wells contained macrophages with no bacteria added (media). Other wells contained macrophages cultured with LVS grown in various bacterial media as indicated: MH, Mueller Hinton broth; CDM, chemically defined medium, which contains 200 $\mu$ M spermine; MH+SP, MH with 200 $\mu$ M spermine.

(B) Titration of spermine levels. *F. tularensis* LVS was grown overnight in MH broth supplemented with various concentrations of spermine (0, 0.2, 2, 20, and 200  $\mu$ M) before inoculating macrophage cultures. For both panels, TNF $\alpha$  levels were measured in supernatants by ELISA and data are presented as mean  $\pm$  SD of triplicate wells within one experiment. Data are representative of 5 (A) and 2 (B) experiments using different blood donors.

### **3.4.2 *Francisella* gene expression changes in response to spermine:**

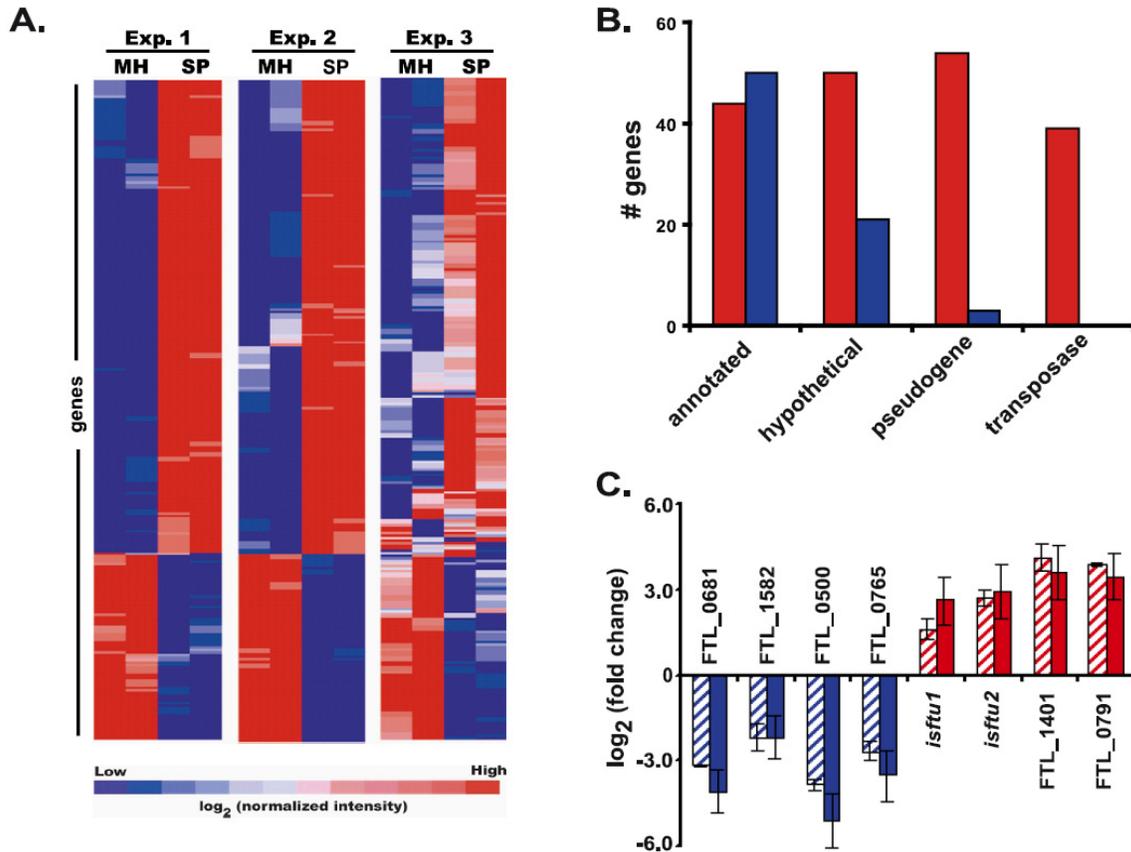
The profound change in cytokine induction suggested that substantial changes occurred in LVS in response to spermine. We sought to define the bacterial response to spermine by global measurements of gene expression. RNA was isolated from LVS grown in MH broth with or without supplemental spermine (200 $\mu$ M). The RNA was then used to produce labeled cDNA target that was hybridized to a custom *Francisella* microarray. Significant differences in gene expression between LVS grown with and without spermine were identified using a J5 statistical test, which was chosen to limit false positives [184]. Those genes whose expression was significantly altered in response to spermine were input into Gene Pattern [185] for hierarchical clustering (Figure 16A). The clustered data demonstrate a clear pattern of transcriptional regulation in response to extracellular spermine. Expression levels in LVS increased for 187 genes (Table 5) and decreased for 75 genes (Table 6) in response to spermine. This indicates that a substantial portion of the *Francisella* transcriptome changes in response to this environmental cue.

To examine the differentially-regulated genes more closely, we categorized them based on their genomic annotation [32,33]. The categories chosen were: ‘annotated’ – those genes with known and annotated functions; ‘hypothetical’ – genes with unknown function; ‘pseudogenes’ –

genes annotated as pseudogenes for any reason; and ‘transposase’ – genes encoding any of the transposases found in the 109 IS elements located throughout the LVS genome [32]. Up-regulated genes included 44 annotated, 50 hypothetical, 54 pseudogenes, and 39 copies of the *isftu2* transposase (Figure 16B, red bars). Conversely, genes with decreased expression included 51 annotated, 21 hypothetical, only 3 pseudogenes, and no transposases (Figure 16B, blue bars). This analysis was remarkable because a relatively high number of pseudogenes and transposases were expressed in response to spermine. In addition, many of the up-regulated genes were in proximity to IS elements in the chromosome. Ten unique probes were designed by the Agilent software to query all *F. tularensis* LVS IS elements in the genome. Since the sequence of each member of a class of IS elements is identical, it was not possible to generate unique oligonucleotides for every individual member. Nevertheless, the IS elements are likely coordinately regulated because of their sequence identity.

To confirm the microarray results, eight genes were chosen, representing both up-regulated and down-regulated transcripts, for analysis using quantitative real time PCR (Q-PCR). Among the down-regulated genes, a component of the polyamine transport (*pot*) system (*potI*, FTL\_0681), a predicted spermidine synthase (FTL\_0500), and *vacJ* (FTL\_0765) were chosen for validation. The transposase in *isftu2*, a hypothetical protein with homology to *lpxF* of *F. novicida* (FTL\_1401), and an ORF encoding a hypothetical protein specific to *Francisella* (FTL\_0791) were chosen from the list of induced genes. Two other genes of interest with J5 scores just below our cut-off were also examined by Q-PCR. These were the remaining member of the annotated *pot* system, *potF* (FTL\_1582), and the transposase in the other predominant class of IS elements, *isftu1*. All of the genes tested by Q-PCR exhibited a similar pattern of expression as was seen in the microarray experiments (Figure 16C solid bars = Q-PCR; striped

bars = microarray data), validating the previous microarray results. These results also independently confirmed increased transcription of the transposases in the IS elements, *isftu1* and *isftu2*.



**Figure 16: *Francisella* gene expression changes induced by spermine.**

(A) Hierarchical clustering of genes identified as statistically significant by J5 test across three independent microarray experiments. Fluorescence intensities across the array were normalized by dividing by the median intensity of that array followed by  $\log_2$  transformation. These values from each experiment were input independently into Gene Pattern individually and clustered using the Pearson correlation. The 185 induced genes and 75 repressed genes clustered together in GenePattern, validating the statistical analysis from GEDA.

(B) Categorical summary of spermine-induced changes in gene expression.

Genes exhibiting increased (red bars) or decreased (blue bars) transcription levels were grouped into functional categories based on their NCBI annotation. Category definitions; ‘annotated’ –genes with known or predicted functions; ‘hypothetical’ – genes with unknown function; ‘pseudogenes’ – genes annotated as pseudogenes for due to mutation, insertion of an IS element, or an unspecified reason,; and ‘IS element’ – indicating any of the 109 annotated transposases found in the LVS genome. Data are presented as total number of genes per category and are derived from the three independent microarray experiments (Panel A).

(C) Gene expression changes measured by Q-PCR. RNA from two of the microarray experiments depicted in panel A and two independent experiments were tested for specific genes using real time PCR. The results of Q-PCR are represented with solid bars, while values from microarray experiments are represented with striped bars. Data for both microarrays and real time are presented as mean  $\pm$  SEM from three individual experiments.

**Table 5: *F. tularensis* genes exhibiting increased expression in response to spermine<sup>a</sup>**

FTL#	GN <sup>b</sup>	J5 <sup>c</sup>	FC <sup>d</sup>	FTL#	GN	J5	FC
FTL_1813	NA <sup>e</sup>	4.9	19.8	FTL_0303	NA	2.8	7.3
FTL_0471	NA	4.7	21.8	FTL_0774	NA	2.8	4.5
FTL_0909	NA	4.7	26.4	FTL_0006	<i>ipdC</i>	2.8	5.2
FTL_0163	NA	4.6	22.2	FTL_1884	NA	2.8	5.7
FTL_1456	NA	4.4	6.2	FTL_1209	NA	2.7	3.1
FTL_0340	NA	4.3	14.8	FTL_0557	NA	2.7	2.9
FTL_0734	NA	4.2	20.0	FTL_0227	<i>frr</i>	2.7	7.1
FTL_1691	NA	4.2	14.7	FTL_0365	NA	2.7	6.0
FTL_0772	NA	4.2	7.5	FTL_1457	NA	2.7	4.6
FTL_0446	NA	4.1	15.5	FTL_1761	NA	2.7	4.1
FTL_0390	<i>pilV</i>	4.0	17.3	FTL_0826	NA	2.6	6.7
FTL_1326	NA	4.0	12.5	FTL_0184	NA	2.6	5.3
FTL_1371	NA	3.8	12.4	FTL_1698	NA	2.6	3.6
FTL_0342	NA	3.7	6.6	FTL_1301	<i>isftu2</i>	2.6	7.3
FTL_0442	NA	3.7	9.7	FTL_0271	NA	2.6	5.5
FTL_1104	NA	3.6	11.7	FTL_1844	<i>gatC</i>	2.6	4.7
FTL_1778	NA	3.6	10.2	FTL_1926	<i>isftu2</i>	2.6	7.4
FTL_1857	NA	3.6	6.1	FTL_1270	NA	2.6	6.6
FTL_1846	NA	3.5	5.2	FTL_0742	<i>lysR</i>	2.6	6.2
FTL_1260	NA	3.5	5.1	FTL_0061	NA	2.6	3.6
FTL_1403	NA	3.4	7.0	FTL_0900	NA	2.6	3.2
FTL_0352	NA	3.4	6.6	FTL_0554	<i>rnc</i>	2.6	5.9
FTL_1098	NA	3.4	10.1	FTL_1768	NA	2.6	5.1
FTL_0158	NA	3.4	9.9	FTL_0575	<i>isftu2</i>	2.6	6.6
FTL_1734	<i>xerD</i>	3.3	6.4	FTL_1618	NA	2.6	5.1
FTL_0889	NA	3.3	8.7	FTL_0911	NA	2.6	6.6
FTL_1518	NA	3.2	9.7	FTL_1732	NA	2.5	4.5
FTL_0555	<i>truB</i>	3.2	11.3	FTL_1572	<i>isftu2</i>	2.5	6.2
FTL_0055	NA	3.2	9.3	FTL_0791	NA	2.5	1.8
FTL_1289	NA	3.1	4.4	FTL_0292	NA	2.5	6.3
FTL_1653	<i>oppA</i>	3.1	6.5	FTL_0819	NA	2.5	2.9
FTL_1470	NA	3.1	8.6	FTL_1847	NA	2.5	5.9
FTL_1651	NA	3.1	4.4	FTL_0162	NA	2.5	4.9
FTL_1777	NA	3.1	6.9	FTL_1919	NA	2.5	4.1
FTL_0912	NA	3.1	8.4	FTL_1652	NA	2.5	3.3
FTL_0565	NA	3.0	7.5	FTL_1112	<i>isftu2</i>	2.5	6.1
FTL_0735	NA	2.9	6.2	FTL_1087	<i>isftu2</i>	2.5	5.9
FTL_1771	<i>pilT</i>	2.9	8.4	FTL_1348	NA	2.5	6.1
FTL_1011	NA	2.9	7.7	FTL_0042	<i>isftu2</i>	2.5	6.0
FTL_1631	NA	2.9	5.5	FTL_0868	<i>isftu2</i>	2.5	5.9
FTL_1008	NA	2.9	8.4	FTL_1319	<i>isftu2</i>	2.5	5.7
FTL_0773	NA	2.9	2.9	FTL_0501	NA	2.5	5.1
FTL_0053	NA	2.9	7.6	FTL_0757	NA	2.5	4.7
FTL_1675	NA	2.9	3.3	FTL_0468	<i>isftu2</i>	2.5	5.8
FTL_1214	NA	2.9	5.9	FTL_1445	<i>isftu2</i>	2.4	5.7
FTL_1814	NA	2.9	3.2	FTL_0441	<i>isftu2</i>	2.4	6.1
FTL_1401	NA	2.8	3.3	FTL_0302	<i>isftu2</i>	2.4	6.0
FTL_0062	NA	2.8	6.1	FTL_1386	<i>isftu2</i>	2.4	6.1

| FTL\_0910 NA 2.8 7.7 | | FTL\_1655 *isftu2* 2.4 5.3 |

<sup>a</sup> – Data are organized by statistical significance      <sup>d</sup> – FC = fold change  
<sup>b</sup> – GN = gene name; based on ENTREgene and [32]      <sup>e</sup> – NA = gene name not available  
<sup>c</sup> – J5 = statistical test score

**Table 6: *F. tularensis* genes exhibiting decreased expression in response to spermine<sup>a</sup>**

FTL#	GN <sup>b</sup>	J5 <sup>c</sup>	FC <sup>d</sup>	FTL#	GN	J5	FC
FTL_0569	NA <sup>e</sup>	-3.4	-10.6	FTL_0500	<i>speE</i>	-2.4	-6.0
FTL_0879	<i>penP</i>	-3.4	-14.8	FTL_1852	NA	-2.4	-6.3
FTL_0815	NA	-3.4	-12.0	FTL_0837	<i>abcd</i>	-2.4	-5.6
FTL_0664	NA	-3.4	-9.1	FTL_1135	NA	-2.4	-5.9
FTL_1528	NA	-3.3	-10.7	FTL_1147	<i>pgk</i>	-2.4	-5.6
FTL_1312	<i>dcuC</i>	-3.2	-9.8	FTL_0097	NA	-2.4	-5.5
FTL_0627	<i>rhtB</i>	-3.1	-9.2	FTL_1568	<i>lysR</i>	-2.4	-5.2
FTL_0478	<i>gcvH</i>	-3.1	-7.3	FTL_0473	NA	-2.4	-5.4
FTL_0671	NA	-3.1	-7.1	FTL_1149	<i>fbaA</i>	-2.4	-6.0
FTL_0280	<i>ansP</i>	-3.0	-12.9	FTL_0814	NA	-2.3	-5.1
FTL_1790	<i>ampG</i>	-3.0	-8.8	FTL_0923	<i>grxB</i>	-2.3	-5.0
FTL_1173	NA	-3.0	-8.3	FTL_0026	NA	-2.3	-5.0
FTL_0675	NA	-2.9	-7.8	FTL_0477	<i>gcvT</i>	-2.2	-5.1
FTL_0816	NA	-2.9	-8.4	FTL_1641	NA	-2.2	-5.8
FTL_1157	<i>iglA</i>	-2.8	-5.9	FTL_1224	NA	-2.2	-4.7
FTL_0348	NA	-2.8	-7.4	FTL_0279	NA	-2.2	-5.7
FTL_0673	<i>panC</i>	-2.8	-6.6	FTL_1164	NA	-2.2	-3.7
FTL_0672	<i>panD</i>	-2.8	-7.0	FTL_0924	NA	-2.2	-4.7
FTL_0881	NA	-2.7	-6.7	FTL_0325	<i>ompA</i>	-2.2	-5.4
FTL_0679	<i>potI</i>	-2.7	-7.8	FTL_0743	<i>fabG</i>	-2.2	-6.2
FTL_0674	<i>panB</i>	-2.7	-5.8	FTL_0681	<i>potG</i>	-2.2	-7.7
FTL_1012	NA	-2.7	-7.6	FTL_1345	NA	-2.2	-5.5
FTL_1219	<i>bioF</i>	-2.7	-6.0	FTL_0703	<i>glyA</i>	-2.2	-4.9
FTL_0382	<i>lysP</i>	-2.6	-7.1	FTL_1522	<i>bioF</i>	-2.1	-4.5
FTL_1167	NA	-2.6	-6.6	FTL_1146	<i>gapA</i>	-2.1	-4.9
FTL_0449	NA	-2.6	-5.2	FTL_0765	<i>vacJ</i>	-2.1	-3.6
FTL_1013	NA	-2.5	-5.8	FTL_0695	<i>mce</i>	-2.1	-4.8
FTL_1159	<i>iglC</i>	-2.5	-5.6	FTL_0583	<i>paaJ</i>	-2.1	-4.4
FTL_0585	<i>caiA</i>	-2.5	-5.6	FTL_1166	NA	-2.1	-3.2
FTL_1165	NA	-2.5	-6.3	FTL_0479	<i>gcvP1</i>	-2.1	-4.2
FTL_0082	<i>cybB</i>	-2.5	-6.0	FTL_0680	<i>potH</i>	-2.0	-4.9
FTL_0694	NA	-2.5	-5.5	FTL_1160	<i>iglD</i>	-2.0	-3.2
FTL_0282	NA	-2.5	-8.6	FTL_0863	NA	-2.0	-4.7
FTL_0499	<i>speH</i>	-2.5	-9.9	FTL_1789	<i>gltA</i>	-2.0	-4.1
FTL_1218	NA	-2.5	-5.2	FTL_0480	<i>gcvP2</i>	-2.0	-3.9
FTL_0379	NA	-2.4	-5.8	FTL_0834	<i>sseA</i>	-2.0	-4.0
FTL_1225	NA	-2.4	-5.6	FTL_1868	<i>emrD</i>	-2.0	-3.7

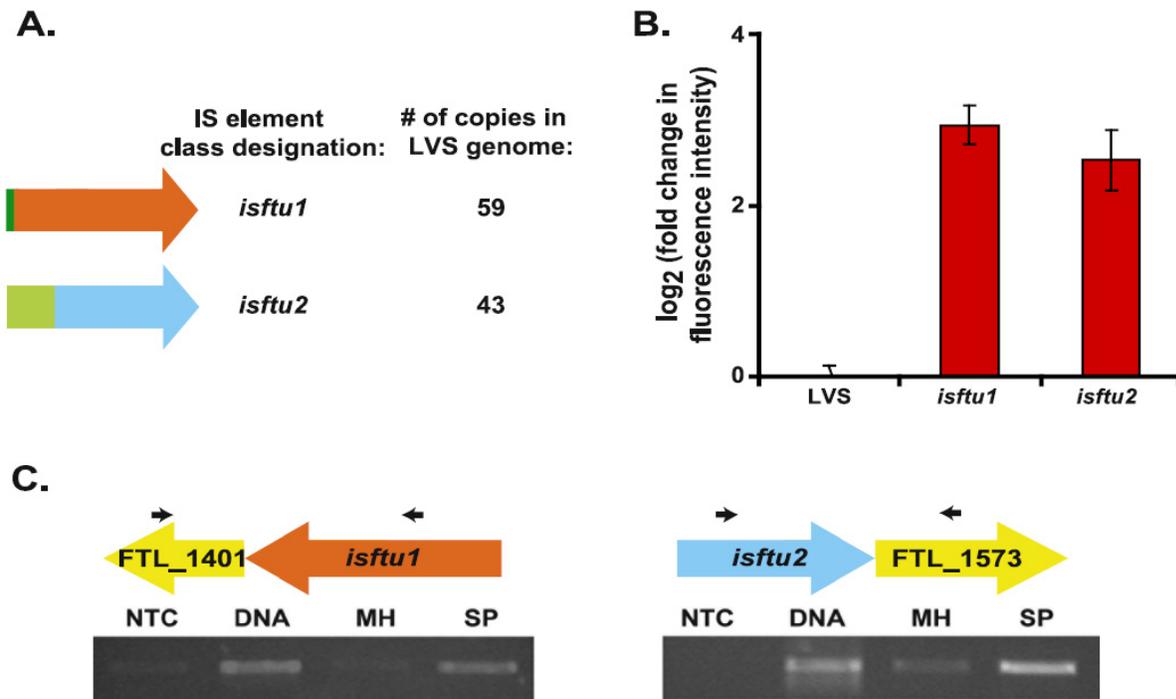
<sup>a</sup> – Data are organized by statistical significance      <sup>d</sup> – FC = fold change  
<sup>b</sup> – GN = gene name; based on ENTREgene and [32]      <sup>e</sup> – NA = gene name not available  
<sup>c</sup> – J5 = statistical test score

### 3.4.3 Role of *Francisella* IS elements in the response to spermine:

Since the expression of a large number of transposases increased in response to spermine, we investigated transcriptional regulation by the IS elements. Reporter constructs were created in which the sequence of a single copy of either *isftu1* or *isftu2* was cloned upstream of a promoter-less red fluorescent protein allele, tdTomato [190]. The IS sequences used for these experiments included the annotated transposase ORF and upstream sequence that was conserved among multiple *isftu1* (30 bp) or *isftu2* (300bp) copies in the LVS genome (Figure 17A). The *isftu*-tdTomato fusions were cloned into pFNLTP8 and transferred to LVS [193]. Fluorescence levels were then examined following incubation with spermine. When *F. tularensis* LVS carrying these reporter constructs was grown in the presence of spermine, a 6-8 fold induction of fluorescence was observed (Figure 17B). Reporter constructs driven by either *isftu1* or *isftu2* behaved similarly, as predicted by the gene expression experiments. In contrast, neither LVS alone nor LVS carrying a promoter-less control vector were fluorescent after incubation with spermine.

While the reporter constructs confirmed transcriptional activation of the IS element and a red fluorescent protein allele, it was unclear if the IS elements directly regulated expression of endogenous genes in the LVS chromosome. One possible mechanism for such an event would be the production of a polycistronic message emanating from the IS element that includes the downstream gene. To examine this, we performed RT-PCR spanning the sequence of two genes shown to be regulated by spermine, FTL\_1401 and FTL\_1573, and the IS elements (*isftu1* and *isftu2*, respectively) located directly upstream in the *F. tularensis* LVS genome (Figure 17C, upper panels). RT-PCR reactions spanning across the genes and their respective IS elements produced the predicted band (~850bp) (Figure 17C, lower panels). In addition to showing

FTL\_1401 and FTL\_1573 were co-transcribed with their upstream IS elements, this semi-quantitative technique also showed increased levels of the polycistronic messages in LVS grown in the presence of spermine. Together with the microarray and Q-PCR data, these results show that the *F. tularensis* IS elements harbor spermine-responsive promoters.



**Figure 17: *Francisella* IS elements contain spermine-responsive promoters.**

(A) Schematic representation of the two primary classes of *F. tularensis* IS elements. Transposase ORFs are indicated with colored arrows. Dark or light green boxes represent the conserved sequence found upstream of 10 copies of each element randomly sampled from the genome: *isftu1*, 30 bp; *isftu2*, 300 bp.

(B) Spermine induces expression of tdTomato using *isftu1* or *isftu2* as promoter elements. *F. tularensis* LVS carrying either *isftu1* or *isftu2* (i.e. the transposase and 5' conserved sequence depicted in panel A) cloned in front of tdTomato was grown in either MH broth or MH broth supplemented with 200μM spermine. Data are presented as mean ± SEM of log<sub>2</sub> (normalized fluorescence intensity MH+ spermine / normalized fluorescence intensity MH) of

six independent transformants read in triplicate (18 total measurements per experiment). These data are representative of over 25 *F. tularensis* LVS transformants harboring each reporter construct.

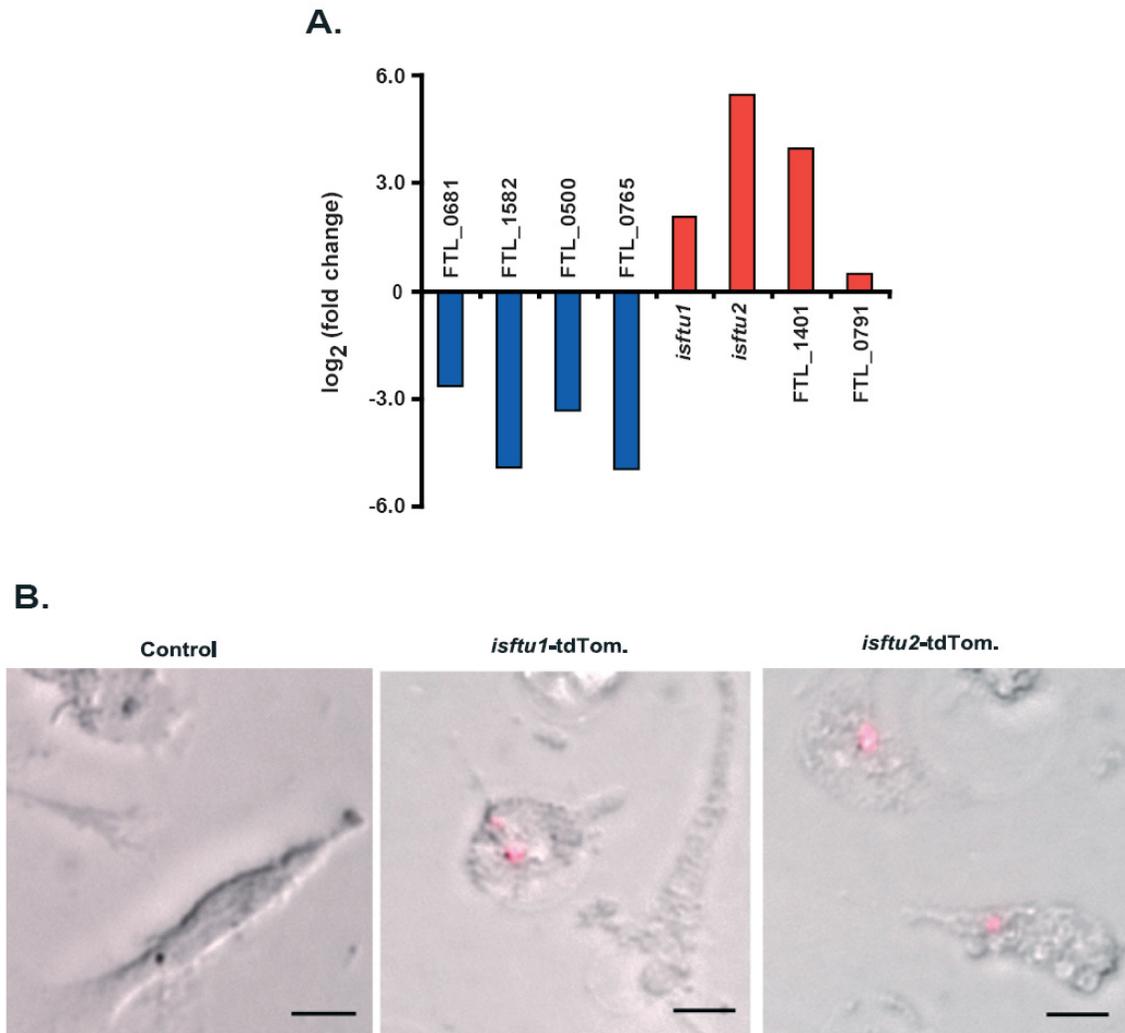
(C) Identification of polycistronic mRNA containing IS elements and downstream genes. Upper panels depict the genomic organization of the IS elements and downstream genes used in this study. Black arrows illustrate the location of primers used for RT-PCR reactions. Primers were designed to generate amplicons across the transposases within the IS elements and open reading frames 3' to the IS elements. Lower panels: agarose gels stained with ethidium bromide showing amplicons from RT-PCR reactions. RNA was derived from LVS following growth in broth culture. NTC = no template control; DNA = positive control using LVS genomic DNA as a template; MH = Mueller Hinton broth; SP = MH with 200 $\mu$ M spermine. No bands were observed in control samples run without reverse transcriptase.

#### **3.4.4 Spermine responsive genes are regulated during intramacrophage growth:**

Because spermine is present at millimolar levels in the cytosol of eukaryotic cells [135], we hypothesized that the macrophage environment would activate transcription of spermine responsive genes. To test this hypothesis we utilized the murine cell line RAW264.7 because the number of primary human macrophages was limiting. LVS was grown within RAW264.7 cells for 48 hours, bacterial RNA was isolated, and gene expression levels were measured by Q-PCR. Patterns of gene expression changes induced by intramacrophage growth (Figure 18A) closely resembled those observed for bacteria growth in MH broth supplemented with spermine (Figure 16). While the magnitude of gene expression changes differed slightly in some cases, the overall trends were identical to that observed with LVS cultured in MH plus spermine (Figure 16C and Figure 18A). FTL\_0791 exhibited a smaller change in gene expression in macrophages than in broth media supplemented with spermine. This is likely the result of converging signals present in the macrophage cytosol including reduced levels of iron and glucose, two signals that have

been shown to affect *Francisella* gene expression [12,13]. Nevertheless, these results show that gene expression changes in response to spermine occur during intramacrophage infection.

Since we were unable to perform the Q-PCR studies in human macrophages, we used the spermine responsive reporter constructs described above (Figure 17) to investigate transcription from *Francisella* IS elements in human cells. For these experiments, human macrophages were infected with LVS or strains bearing the reporter constructs and visualized by fluorescence microscopy. Induction of tdTomato expression was observed when the bacteria were located inside macrophages, but not when the bacteria were extracellular (Figure 18B). Similar to observations in broth cultures, neither LVS alone nor LVS with a promoter-less tdTomato construct exhibited fluorescence during macrophage infections (Figure 18B and data not shown). These results confirm that the LVS response to spermine, specifically the activation of a cryptic promoter located within the IS elements, occurs during infection of human macrophages.



**Figure 18: Regulation of spermine responsive gene during intracellular growth.**

(A) LVS gene expression changes induced by growth in RAW264.7 cells were examined using Q-PCR. The data are presented as log<sub>2</sub> transformed fold change comparing LVS grown intracellularly to bacteria grown in broth. These data are representative of two individual macrophage infections that showed similar results.

(B) IS element reporter constructs are activated inside macrophages. Macrophages were infected with LVS carrying the IS element-tdTomato reporter constructs at an MOI of 100 for 2 hours, followed by washing and gentamicin treatment. Macrophages were examined for tdTomato expression at 48 hours post infection using fluorescence microscopy. Scale bars represent 10µm. Similar results were seen in 4 separate experiments.

### 3.4.5 Disruption of the *potF* gene eliminates *Francisella* response to spermine:

To further characterize the interaction between spermine and LVS, we investigated how the bacterium detects spermine in the environment. We studied the *pot* system annotated in LVS genome (FTL\_0679, FTL\_0680, FTL\_0681, and FTL\_1582) as a likely participant. The *pot* system is homologous to the ABC transporter family, members of which have been shown to control gene expression in other systems [140]. To investigate the role of the *pot* system in LVS phenotypes, a gene disruption mutant was constructed in the periplasmic binding protein of this complex, the *potF* gene locus (FTL\_1582). This gene was chosen for disruption as its genomic organization minimizes the possibility of polar effects following insertion (Figure 19A, upper panel). Disruption of *potF* was achieved using a single crossover method (Figure 19A, lower panel) [187], which created a copy of the gene predicted to encode a protein missing the last 30 amino acids, including a conserved residue required for polyamine binding [188]. One mutant colony, *potF-1*, was selected for further investigation. Integration of the vector in the *potF* locus was confirmed by PCR using genomic DNA as a template (Figure 19B). An amplicon from the entire *potF* locus was obtained only when wild-type DNA was used as the template (Figure 19B, upper panel). Alternatively, when primers were utilized to amplify across the insertion vector and through the *potF* locus, a PCR product could only be detected in the mutant bacteria (Figure 19B, lower panel). These data confirm the insertion vector integrated within the *potF* locus. To confirm the loss of protein expression in this mutant we performed an immunoblot to examine levels of PotF in LVS and *potF-1*. Even though significantly more protein was loaded into the *potF-1* lane, no protein expression could be detected in this mutant (Figure 19C). LVS and *potF-1* grew at similar rates in MH cultures (Figure 19D), indicating that *potF* is not an essential gene for growth in broth. Both LVS and *potF-1* grew at similar rates on solid media, but there were

visible differences in colony morphology between the two on solid media. Mutant colonies were characteristically more smooth and glossy compared to wild-type colonies on chocolate agar (Figure 19E).

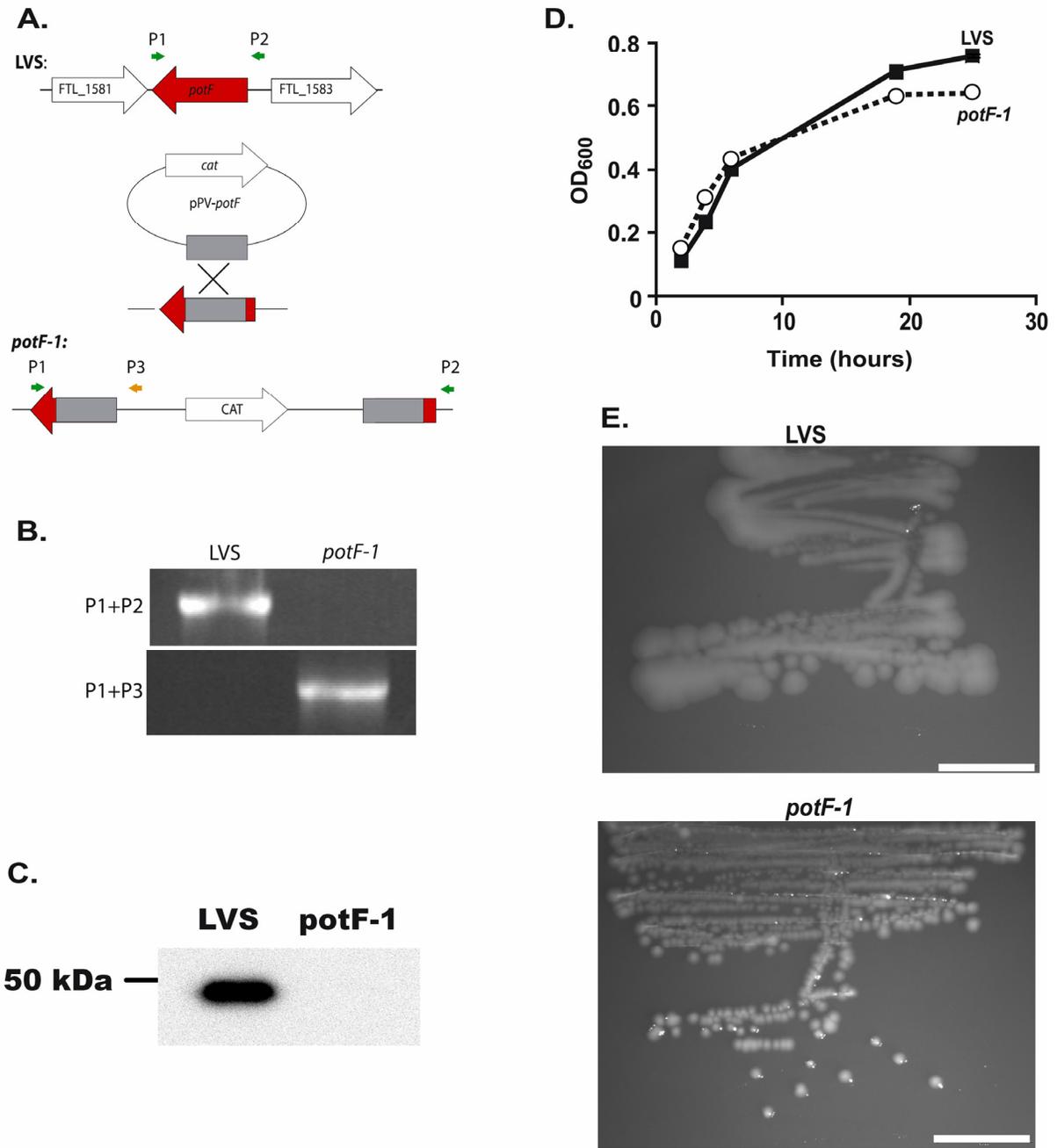


Figure 19: Mutation of *potF* gene in *F. tularensis* LVS.

(A) Strategy for gene disruption using a single cross-over leading to recombination of pPV-potF into the chromosomal *potF* gene. Top panel: genomic organization of *potF* locus in LVS. Grayed area represents the internal 900 bp of *potF* for insertion into the disruption construct (pPV-potF, middle panel). Bottom panel: genomic organization of *potF-1* after integration of *pPV-potF*. Colored arrows represent the location of primers used for PCR confirmation of the mutation below.

(B) PCR confirmation of the *potF* mutation. Upper panel: PCR for the wild-type *potF* locus. Lower panel: PCR reaction to amplify between genomic *potF* and the insertion construct. The primers used for each reaction are indicated in Panel A. Purified genomic DNA from mutant and wild-type LVS was used as the template for these reactions.

(C) PotF immunoblot to confirm loss of protein expression following *potF* disruption. Blots were probed with antibody produced against a PotF homolog from *Streptococcus pneumoniae*. The *potF-1* lane contains approximately three times total protein that is in the LVS lane. Protein loads were quantitated by silver staining.

(D) Growth characteristics of *potF-1* mutant compared to the parent LVS. *F. tularensis* LVS with disrupted copy of the *potF* gene (dotted line) and wild-type LVS (solid line). Bacteria were grown in MH broth at 37°C and shaken at 250rpm. No significant difference was observed if spermine was included (data not shown).

(E) Morphological differences between *potF-1* and wild-type LVS. Bacteria were grown on the same chocolate (II) agar plate for 3 days at 37°C+5%CO<sub>2</sub> before imaging. Pictures were taken with an Olympus MVX10 stereoscope under identical lighting and magnification conditions. No modifications were made to brightness or contrast of these images. Scale bar equals 5mm.

We next examined the ability of the *potF-1* mutant to respond to spermine. Both mutant and wild-type LVS were grown in MH broth with or without supplemental spermine. Macrophages were then exposed to these bacteria and TNF $\alpha$  levels in the culture supernatants were measured. Wild-type LVS induced no detectable cytokine production after growth with spermine (Figure 20A), similar to what was observed previously (Figure 15). In contrast, *potF-1* induced significantly higher levels of TNF $\alpha$  from macrophages (p=0.0002 compared to wild type). The mutant did not respond to supplemental spermine and continued to induce high levels

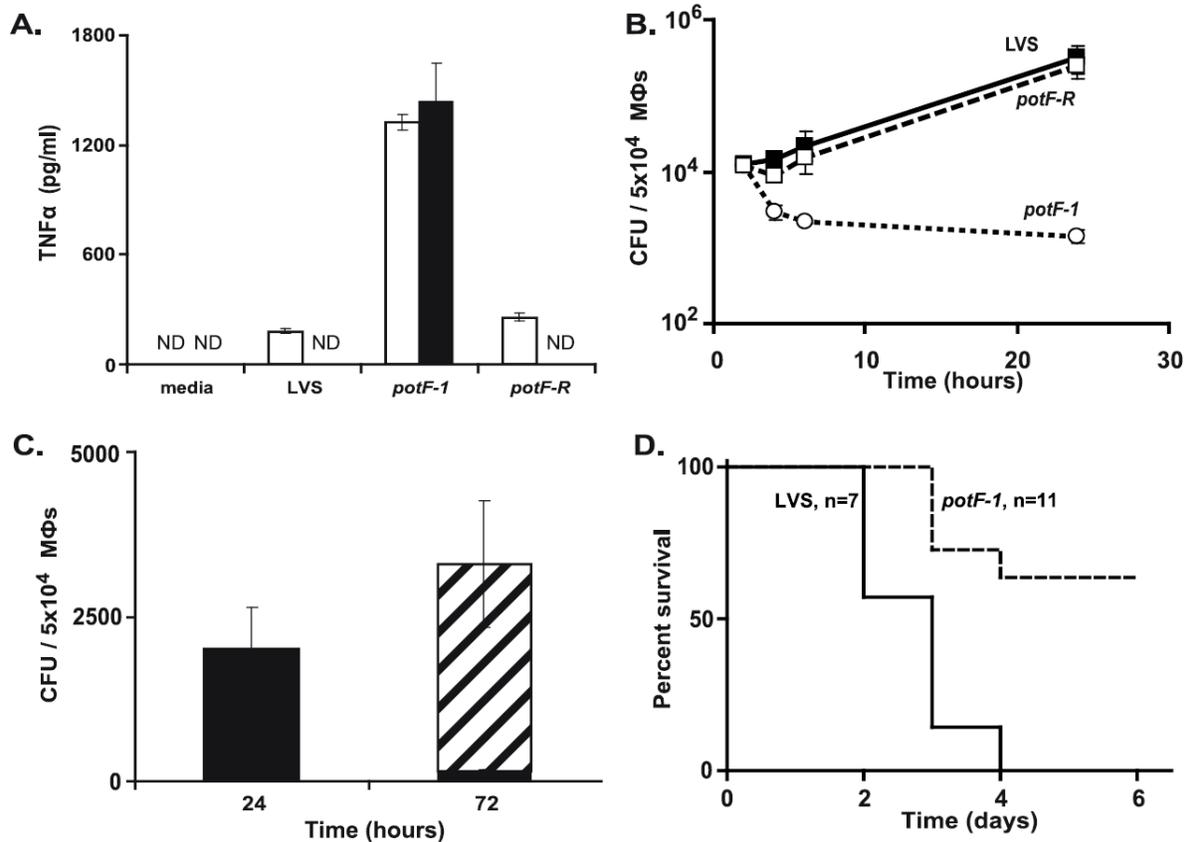
of TNF $\alpha$  from macrophages (Figure 20A). These results indicate that the polyamine transport system is necessary for inhibition of proinflammatory cytokine responses in macrophages exposed to *F. tularensis* LVS grown in spermine.

Since *potF-1* did not respond to extracellular polyamines and TNF $\alpha$  production was markedly increased in macrophages infected with this strain, we hypothesized that this mutant would be attenuated in a macrophage environment. Human macrophages were infected with LVS or *potF-1* and lysed at various time points post infection to enumerate viable bacteria. Wild-type LVS grew nearly 100-fold within macrophages over a 24 hour period (Figure 20B; closed squares). In contrast, intracellular growth of the *potF* mutant was significantly impaired, with nearly a three log<sub>10</sub> difference in CFU after 24 hours compared to wild type (Figure 20B; open circles). In fact, there was rapid killing of *potF-1*, approximately 10-fold, by naïve macrophages within several hours after infection. These data indicate that the ability to detect spermine in an intracellular environment was essential for this organism's survival and growth within host macrophages.

Scrutinizing the colonies derived from macrophage infections revealed a phenotypic shift of *potF-1*. As stated above, *potF-1* colonies were distinct from wild-type LVS colonies on chocolate agar plates (Figure 19C). During the course of our experiments, reversion of the mutant to wild-type appearance was observed following extended incubation with macrophages. Three to six days post infection, the majority of the bacteria isolated from the macrophages were of wild-type appearance even though a pure culture of *potF-1* was inoculated (Figure 20C). This reversion was not simply the result of growth in the absence of antibiotic selection, since *potF-1* grown for 5 days in broth culture without chloramphenicol selection maintained the mutant phenotype with no signs of reversion (data not shown). One of the revertants (*potF-R*) isolated

from macrophages following three days of growth was used for further study. *potF-R* lost chloramphenicol resistance, as expected, and the wild-type *potF* locus was found to be reconstituted when genomic DNA was tested by PCR (data not shown). In functional tests, *potF-R* also exhibited a wild-type phenotype. Macrophages infected with *potF-R* grown in MH broth alone induced TNF $\alpha$  from macrophages, whereas *potF-R* grown in MH with spermine induced no detectable TNF $\alpha$  (Figure 20A). Similarly, *potF-R* grew in macrophages to levels comparable to wild-type LVS (Figure 20B – open squares). These data suggest that the macrophage environment is hostile for the attenuated *potF* mutant and reversion to the wild-type *potF* locus provides a selective advantage to the bacterium.

To further investigate the attenuation of *potF-1*, we utilized a published model of chicken embryo infection [192]. Seven day old embryos were infected with either LVS or *potF-1* and examined daily for six days. Infection with wild-type LVS led to rapid death, with none of the embryos surviving beyond day 4 post infection. In contrast, 60% of those given *potF-1* survived to the end of the experiment (Figure 20D,  $p=0.0019$  by log rank test). This difference in survival further demonstrated the attenuation of *Francisella* lacking a functional polyamine transport system.



**Figure 20: Attenuation of the *F. tularensis* LVS *potF* mutant.**

(A) Disruption of *potF* in *F. tularensis* LVS eliminates the response to extracellular spermine. LVS, *potF-1*, and revertant bacteria were grown in MH broth alone (white bars) or MH broth with 200  $\mu$ M supplemental spermine (black bars). Macrophages were infected with bacteria from these cultures at an MOI of 10 for 24 hours. Media, control wells with no bacteria added. TNF $\alpha$  levels were measured in supernatants by ELISA. Data presented are mean  $\pm$  SD of triplicate wells within one experiment and are representative of 5 experiments using different donors. ND = below detectable levels. Details of mutant construction located in supplementary methods.

(B) *potF-1* growth defect in macrophages. Macrophages were infected with either LVS (■), *potF-1* (□), or *potF-R* (○) at an MOI of 100 for 2 hours, followed by three washes and treatment with gentamicin. Macrophages were lysed at indicated times and intracellular bacteria were enumerated by plating. Data are mean  $\pm$  SD of triplicate wells within one experiment and are representative of 3 experiments performed using cells from separate donors.

(C) Reversion of *potF-1* to wild-type LVS. Macrophages were infected with *potF-1* as described in (A) above. Macrophages were lysed at 24 and 72 hours post-infection and plated for CFU analysis. *potF-1* and revertant bacteria were differentiated based on colony appearance and counted separately. Data are mean  $\pm$  SD of triplicate wells within one experiment and are representative of 3 experiments performed using cells from different donors.

(D) *potF-1* is attenuated in vivo in the chicken embryo model. Embryos were infected with LVS (n = 7 embryos from one experiment,  $4.2 \times 10^6$  bacteria) or with *potF-1* (n = 11 embryos from two experiments,  $8.2 \times 10^6$  and  $5.0 \times 10^6$  bacteria). Results are shown as Kaplan-Meier survival curves, and differences in survival were calculated by the log-rank analysis;  $P = 0.0019^{**}$ .

### 3.5 DISCUSSION:

Although *Francisella tularensis* is a pathogen known to infect many cell types, the mechanisms involved in bacterial adaptation to its intracellular lifestyle have not been elucidated. Here we have reported the identification of a molecule, spermine, exclusively produced by eukaryotic cells [135] that dramatically alters the transcriptome and phenotype of *F. tularensis*. We also present evidence that the same transcriptional changes occur during infection of macrophages, further supporting the idea that *Francisella* recognizes spermine as a signal to this environment.

Our results show that spermine is sufficient to induce substantial changes in global gene expression, including induction of two classes of *Francisella* IS elements, leading to a phenotype that stimulates macrophages poorly. A mutant in a putative polyamine transport system is incapable of responding to spermine, resulting in enhanced stimulation of macrophages. Recognition of intracellular spermine is essential for growth and survival of *F. tularensis* in macrophages, and virulence in an *in vivo* model of infection. Other experiments have shown that agmatine and putrescine are unable to elicit the phenotypic changes seen here with spermine, though high concentrations of spermidine can alter LVS phenotypes (data not shown). Transcription of spermidine biosynthesis genes is upregulated in the absence of supplemental spermine (Table 5 and FTL\_0500 in Figure 16C). It is therefore unlikely that bacterial-derived spermidine can induce the responses observed since gene expression and cytokine induction are vastly different in bacteria grown without supplemental spermine (Figures 15 and 16). Spermine, however, is found at millimolar concentrations inside eukaryotic cells [135], the

normal host environment of *Francisella*. It is, therefore, not surprising that *Francisella* evolved to recognize spermine as a signal of its intracellular environment within the host.

Global gene expression analysis using a comprehensive *Francisella* microarray provided key insights into our studies. One of the more unexpected findings was induction of transposase gene expression in the IS elements, specifically *isftu1* and *isftu2*, by spermine (Figure 16, Figure 18, and Table 5). In addition, many of the non-transposase genes induced by spermine treatment were adjacent to the IS elements in the genome. This relationship led to the hypothesis that the IS elements carry a spermine-responsive promoter. Further experimentation using reporter constructs supported this hypothesis (Figure 17). While control of gene expression by promoters carried on mobile genetic elements has been observed previously [194,195], there are no reports of this occurring to the extent observed here in *Francisella*. The genome sequences of various *Francisella* subspecies contain over 100 such IS elements, however their genomic localization differs between subspecies [32]. Our data suggest that gene regulation in response to spermine will differ between various *Francisellae* and may contribute to the range of virulence phenotypes observed among sub-species [14].

Many of the *F. tularensis* LVS gene expression changes in response to spermine (Figure 16; Tables 5 and 6) are predicted to have significant effects on the *F. tularensis*-macrophage interaction. For example, two genes with decreased expression (FTL\_0695 and FTL\_0765) are associated with events leading to cell entry of other pathogens. FTL\_0695 is a homolog of a mycobacterial protein associated with entry into non-phagocytic cells [196,197]. FTL\_0765 is a homolog of *vacJ* of *Shigella flexneri*, a protein that disrupts cell membranes and is involved in bacterial spread between host cells [198]. It would be advantageous for *Francisella* to repress

these genes/proteins following entry into the cytoplasm to preserve macrophage integrity and sustain the intracellular environment that promotes bacterial growth.

We also observed reduced transcript levels for some genes from the *Francisella* pathogenicity island [9] (Table 6). Specifically, the genes encoding intracellular growth locus (Igl) A, C, and D proteins were all significantly down-regulated in response to extracellular spermine (Table 6). However, we have previously shown IglC levels do not necessarily correlate with transcript levels [108]. Moreover, other signals, such as iron limitation, are also likely to influence protein levels during an infection [12,77]. The gene expression changes we have seen in response to spermine may contribute to the overall fine tuning of IglA, C, and D protein levels to optimize replication of *Francisella* within host cells.

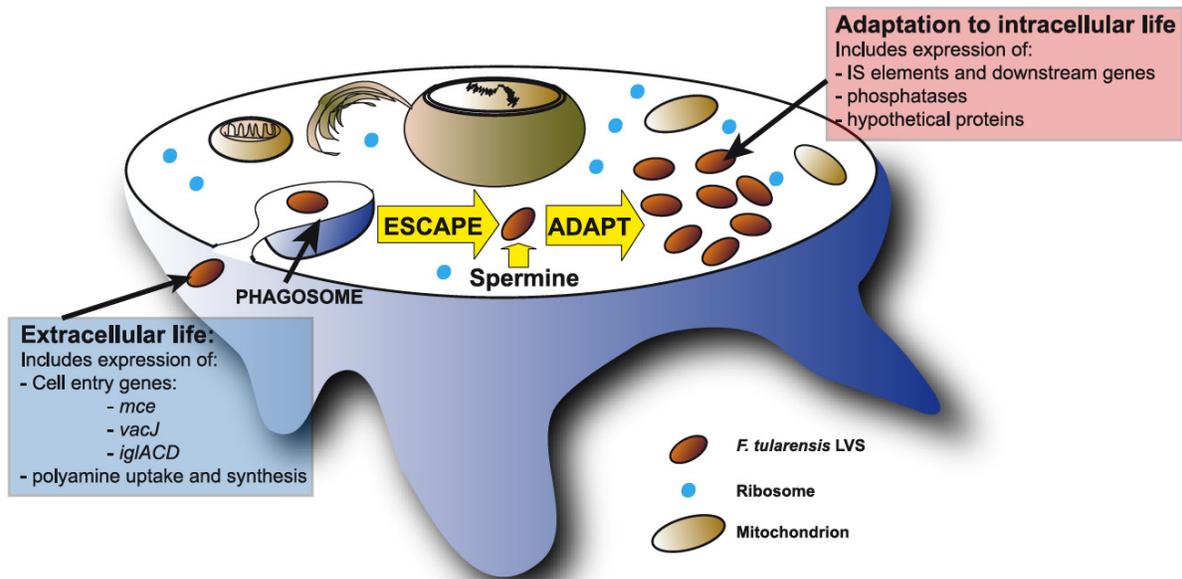
Fewer than 25% of the genes induced by spermine have known functions based on genome annotation (Figure 16B and Table 5) [33]. Among these known genes, we observed an increase in the expression of phosphatases (FTL\_0158, FTL\_0889, and FTL\_1401). Genes encoding various phosphatases have been associated with *Francisella* virulence [83,199] and one in particular, *lpxF*, appears to play a role in inhibition of macrophage responses in *F. novicida* [199]. Many of the genes whose expression was increased in response to spermine are annotated as hypothetical proteins, including some with no gene or protein homologs outside of the *Francisellae* [33]. It is likely that these hypothetical proteins play an important role in the intracellular growth and provides a focused list of potential virulence genes for further study.

The ABC transporter encoded by *potFGHI* is predicted to be involved in spermine uptake. Transcription of the genes encoding this system was decreased following growth with supplemental spermine (Figure 16 and Table 6). This was expected since less transport machinery would be required when extracellular spermine is abundant. Disruption of the

periplasmic binding protein (FTL\_1582) of this complex disrupts the response to extracellular spermine, causing significant attenuation in both human macrophages and chicken embryos (Figure 20). These data, along with the fact that recovery of the *potF* locus restored wild-type phenotypes, demonstrate that this ABC transport system is involved in spermine responsiveness in *F. tularensis* LVS. The regulation of gene expression could be due to transcription factors controlled directly by the ATP binding subunit of the transporter, as seen in *E. coli* [140], or due to interactions of spermine with DNA or RNA [135].

Polyamine production and detection appears to be an emerging theme in host-pathogen interactions. In *Streptococcus pneumoniae*, deletion of the periplasmic binding protein of the *pot* system attenuated the pathogen in mice [151]. Our results shed new insight on this finding and suggest the deletion eliminated *S. pneumoniae* virulence gene regulation controlled by the *pot* system. Vaccination against components of the *pot* system of both *S. pneumoniae* and *Francisella* confers some protection against respective challenge [200,201], supporting the idea that this transport system is necessary during infection. It is also of interest that host polyamine synthesis pathways are activated by pathogens. Infection of mice with *F. tularensis* subsp. *tularensis* [202] and murine macrophages with *Bacillus anthracis* [203] increases expression of host ornithine decarboxylase. This enzyme catalyzes a key early step in polyamine synthesis and is likely to result in spermine accumulation, further enhancing pathogen virulence. In anthrax infections, increased host ornithine decarboxylase activity is associated with suppression of apoptosis in infected macrophages, thereby preserving the pathogen's niche [203]. These observations, along with those presented in our study, suggest pathogen responses to polyamines may be a common regulator of virulence and a potential target for therapeutics.

Our results suggest a model in which spermine found in the macrophage cytosol elicits substantial changes in the *Francisella* phenotype (Figure 21). *Francisella* is known to be taken up into phagocytic vesicles [4] only to escape into the cytoplasm within a few hours [4,52,54]. In our model, the bacterium encounters spermine once it escapes from a phagosome and reaches the cytoplasm. The spermine triggers LVS through the polyamine transport system (*potFGHI*) to alter its gene expression profile with the subsequent gene expression changes controlled, in part, by the many IS elements present in the *Francisella* genome. Wild-type LVS grown without spermine elicits relatively low levels of cytokines compared to the *potF-1* mutant (Figure 20A). Our model predicts that these wild-type bacteria are adapting in response to the spermine they encounter in the macrophages, thereby blunting the total amount of cytokine induced. Therefore, the phenotype of intracellular LVS is characterized by inhibition of macrophage cytokine responses [108], intracellular survival, and intracellular proliferation (Figure 20). Though additional mechanistic details by which the *pot* system regulates transcription from these IS elements and other sites in *Francisella* remain to be determined, the end result enables the bacterium to adapt to its new environment. Taken together, these data define a complex system of gene regulation in response to an intracellular environment that contributes to the overall success of this pathogen. Because other intracellular pathogens have polyamine transporters homologous to the *pot* system investigated here, the adaptive response we have characterized for LVS may be broadly relevant for bacterial pathogenesis.



**Figure 21: Model of *Francisella* response to spermine in macrophages.**

*Francisella* is phagocytosed by macrophages and escapes into the cytoplasm, where it encounters spermine. In response to this signal, *Francisella* down-regulates many genes, including those involved in entry into host cells and polyamine synthesis and acquisition. Simultaneously, the bacterium up-regulates other genes, including those downstream of its many IS elements, to adapt to the intracellular environment.

#### **4.0 CHAPTER 3: GLOBAL EFFECT OF CULTURE CONDITIONS ON *FRANCISELLA TULARENSIS* GENE EXPRESSION.**

##### **4.1 ABSTRACT**

Although a variety of media are available to cultivate *Francisella tularensis*, the impact of these different growth environments on host-pathogen interactions has not been investigated systematically. We have previously shown that growth in different culture media alters *F. tularensis* phenotypes and the host responses the bacteria elicit. Here we conduct a comprehensive analysis of gene expression and virulence traits in bacteria cultivated in different environments. We have found that bacteria grown in a chemically defined medium, modified Mueller Hinton broth, or Tryptic Soy Broth exhibit dramatically altered phenotypes. We found that the bacteria from these broths stimulate macrophages differently, with LVS grown in MH inducing the most cytokine production. The level of IglC protein also varies depending on the culture media. Because LVS grown in these various media exhibit such differing phenotypes, we sought to understand the changes induced in the bacteria following growth in each. Using microarray analysis, we have identified over 400 genes that have altered expression patterns following growth in these conditions. Importantly, conditions associated with low macrophage stimulation have been associated with specific changes in transcriptional regulation. The range of differences between the bacteria grown in these media suggests that care must be taken in

experimental design. A better understanding of the effects of these common media on *Francisella* is necessary because the resulting bacterial phenotypes vary significantly. Together, the results should provide valuable insights into *Francisella* physiology.

## 4.2 INTRODUCTION

*Francisella tularensis* is a highly pathogenic gram-negative bacterium, and the causative agent of tularemia [1]. Members of the *Francisellae* are facultative intracellular bacteria that are primarily associated with growth inside macrophages, though they are also known to replicate in other cell types including endothelial cells, epithelial cells, dendritic cells, and hepatocytes [51]. *Francisella* subspecies have been associated with over 190 mammalian species as well as birds, reptiles, and fish [3]. These bacteria are also carried and transmitted by a wide range of arthropods, including ticks, mites, and mosquitoes. Environmental *Francisella* has also been associated with aquatic environments, including the ability to replicate within amoeba [3,71].

Since it is capable of growth and survival in such a large range of environments, it is vital that the bacterium be able to recognize these conditions, allowing effective adaptation to its current environment. *Francisella* is known to adapt to the host intracellular environment. The *Francisella* pathogenicity island gene, intracellular growth locus (*igl*) C was first identified as a 23kDa protein up-regulated by growth in macrophages [77]. Macrophage growth has also been associated with phenotypic changes in *Francisella* with bacteria grown intracellularly eliciting minimal cytokine response on subsequent macrophage infection [132]. Further studies have identified oxidative stress, low iron concentrations, and low glucose levels, conditions that would be encountered in a host, as stimuli leading to the regulation of gene and protein expression in these bacteria [12,13,129-131].

We have previously shown bacterial phenotypes specific to growth medium used to cultivate the organisms [108]. The use of a chemically defined medium (CDM) leads to bacteria

that elicit significantly less cytokine production from macrophages than those grown in modified Mueller Hinton (MH) broth [108]. Further study identified a role for a single component in the CDM, spermine, that was responsible for the phenotype previously examined (see Chapter 2). With these data in mind, we sought to examine the differences in *Francisella* gene expression induced solely due to different media conditions used for bacterial growth. Here, we have compared bacteria grown in three *Francisella* media; CDM, MH, and tryptic soy broth with supplemental cysteine (TSBc). These media are commonly used in laboratories throughout the world to culture *Francisella*, though their effect on the bacteria has not been clearly defined. We show significant differences in the macrophage response to bacteria grown under these conditions, as well as differences in expression of the known virulence factor, IglC. A global comparison of gene expression across these conditions identified major differences induced in the *Francisella* transcriptome. These data have broad implications to the field as they indicate that media choice may influence the outcome of experiments examining this bacterium.

### 4.3 MATERIALS AND METHODS

Francisella strains and cultivation: *F. tularensis* LVS was kindly provided by Dr. Karen Elkins (U.S. Food and Drug Administration). For all experiments, LVS was initially grown on chocolate II agar for 1-3 days at 37°C and 5% CO<sub>2</sub>. Bacteria were then cultivated in either Mueller-Hinton broth (supplemented with 0.1% glucose, 0.025% ferric pyrophosphate (Sigma), and Isovitalex (Becton Dickinson)), a chemically defined media [158], or tryptic soy broth supplemented with 0.01% cysteine.

Macrophage culture and infection:

Macrophage cultures were performed as described previously [108]. Overnight cultures of LVS were centrifuged, washed twice in DMEM and resuspended in infection media (DMEM supplemented with 1% human serum, 25mM HEPES (Invitrogen), and 1X Glutamax (Invitrogen)). Bacteria were then diluted to a multiplicity of infection (MOI) of 10 and co-incubated with macrophages for 24 hours. All MOI were confirmed by plating for viable cfu. All use of human-derived cells was approved by the University of Pittsburgh Institutional Review Board.

ELISA analysis:

ELISA analysis was performed as previously described [108]. Macrophage supernatants, harvested 24 hours following the introduction of bacteria, were subjected to ELISA analysis to measure TNF $\alpha$  and IL-12 p40 production (R&D Systems). Following the addition of TMB

substrate solution (Dako) and measurement of optical density using a Molecular Dynamics M2 plate reader, cytokine concentrations were calculated from a standard curve. The limit of detection for the both TNF $\alpha$  and IL-12 p40 ELISAs was 15 pg/ml.

#### Protein Electrophoresis and Immunoblotting:

Ig1C immunoblots were performed using overnight bacterial broth cultures. Cultures were normalized based on bacterial numbers and approximately  $2 \times 10^9$  bacteria were pelleted, washed, resuspended in 100  $\mu$ l of LDS loading buffer (Invitrogen) with  $\beta$ -mercaptoethanol, and boiled for 5 minutes. Eight  $\mu$ l of whole cell lysate were separated on a on a 4-12% Bis-Tris gradient gel (Invitrogen). Separated material was transferred to PVDF membrane using XCell II<sup>TM</sup> Blot Modules (Invitrogen), according to manufacturer's protocol. Following transfer and blocking with 5% (wt/vol) non-fat dry milk in TBST, membranes were probed with a mouse anti-Ig1C monoclonal antibody (clone 10D12, 1:20 dilution of hybridoma supernatant, Immuno-Precise Antibodies Ltd.) and a rabbit anti-hsp70 polyclonal antibody (1:750, GeneTex, Inc.). Following incubation with secondary antibodies, anti-mouse IgG antibody conjugated to HRP (1:1000, Sigma) and anti-rabbit IgG antibody conjugated to HRP (1:1000, Sigma), proteins were visualized using ECL chemiluminescent reagent (Amersham Biosciences) and exposure to film. Fold difference in protein amounts were calculated by comparing integrated density values (IDV) a BioRad Gel Doc system and Quantity One software (V4.4.0) ( $IDV = \sum(\text{each pixel value} - \text{background pixel value})$ ) and normalizing to Hsp70 expression.

#### RNA harvest and analysis:

Five ml of broth culture was mixed immediately with 15 ml TriReagent LS (Molecular Research Center). After adding 4 ml chloroform, the aqueous phase was separated by centrifugation in a Phase Lock Heavy tube (Eppendorf). RNA was precipitated with isopropanol, followed by centrifugation. Pellets were washed with 80% ethanol and resuspended in nuclease-free water. Fifty  $\mu\text{g}$  of the RNA-containing mixture was treated with DNase (Turbo DNA-free, Ambion), followed by ammonium acetate precipitation. RNA quantity was measured by spectrophotometry and quality with an Agilent Bioanalyzer.

#### Microarray design:

Custom *Francisella* microarrays were designed using Agilent's eArray framework. All ORFs, including pseudogenes, were included for *F. tularensis* spp. *tularensis* (SchuS4), *F. tularensis* spp. *holarctica* LVS, *F. tularensis* spp. *holarctica* OSU18, *F. novicida* U112 and the *Francisella* plasmids, pOM1, and pFNL10. Each gene from SchuS4, LVS, and OSU18 was spotted in duplicate on the array, while the others were included as single copies.

#### Microarray target preparation and hybridization:

Synthesis of labeled cDNA target was performed using 10  $\mu\text{g}$  of total RNA, 0.5  $\mu\text{g}$  random hexamers (Invitrogen), and MMLV (Agilent). cDNA target was labeled with Alexa Fluor 555 conjugated dUTP according to manufacturer's protocol (AF555-aha-dUTP; Invitrogen). Reactions were incubated at 40°C for 3 hours. Following cDNA synthesis, remaining RNA was hydrolyzed with 10 $\mu\text{l}$  1N NaOH and 10 $\mu\text{l}$  0.5M EDTA. pH was then neutralized with 10  $\mu\text{l}$  1M HCl and the labeled cDNA was precipitated using ammonium acetate/isopropanol and washed with 80% ethanol. 0.5 $\mu\text{g}$  of labeled cDNA was hybridized to custom Agilent 8 x 15K

microarrays according to the manufacturer's protocol and incubated at 60°C for 18 hours in a rotary oven. Following hybridization, arrays were washed with Agilent wash buffers before being scanned on an Agilent microarray scanner.

#### Microarray data analysis:

Analysis of the microarray data was carried out using National Institute of Aging (NIA) array analysis tools (NIA array analysis; <http://lgsun.grc.nia.nih.gov/ANOVA/>) [204]. Briefly, these data were median normalized and  $\log_2$  transformed prior to input into the NIA software package. Data were grouped into categories, MH, CDM, or TSB-c, and subjected to an ANOVA analysis in order to compare the data among the three categories. Stringent settings were used for the ANOVA test including: z-value=8; false discovery rate = 0.05; error model = maximum of averaged and actual error variance [204]. This test identified genes unique to each media type. Tables 7 and 8 report the *Francisella* gene identification number for each gene uniquely up or down-regulated following growth in a given media, based on the ANOVA analysis. Genes meeting these requirements were then input into GenePattern [185] and data are presented as a hierarchical clustering (Pearson correlation) of  $\log_2$  transformed normalized intensity values for these genes.

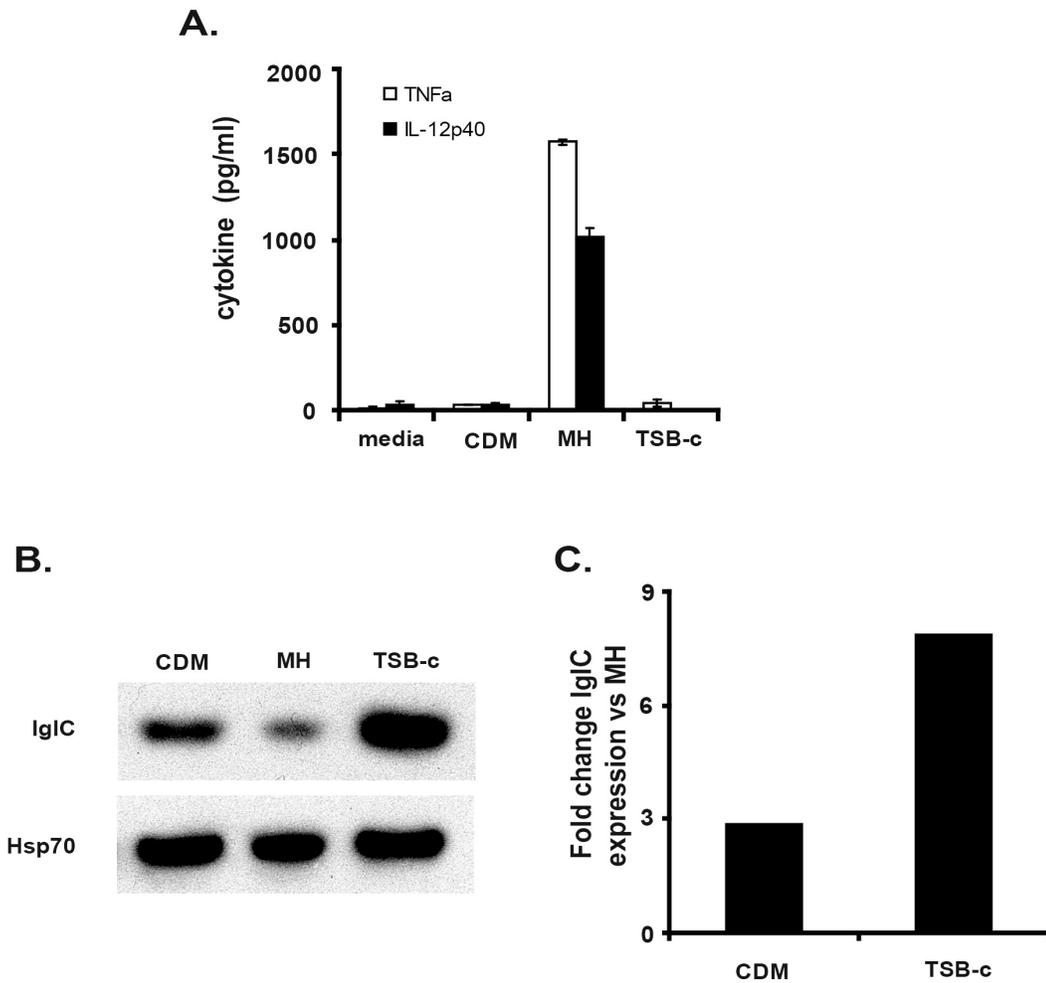
## 4.4 RESULTS AND DISCUSSION

### 4.4.1 Effect of growth conditions on *F. tularensis* LVS:

MH broth is commonly used to culture the bacteria prior to inoculation of *in vitro* cell cultures for studies examining *Francisella* activation of immune cells . These reports have concluded that *Francisella* induces activation of a variety of cells including macrophages, dendritic cells, and epithelial cells [5,92,107,132,156,167]. Conflicting reports have shown that *Francisella* does not induce cytokine production, and also inhibits cellular activation through Toll-like receptors (TLRs) following the addition of lipopolysaccharide to *Francisella*-infected macrophages [127,128]. Previous studies in our laboratory have demonstrated differences in bacterial phenotypes following growth in various culture conditions [108]. To evaluate the potential role of varying culture conditions on *Francisella* phenotypes more systemically, we analyzed the macrophage:bacteria interaction using LVS grown in the three culture media commonly utilized to cultivate *Francisella*; CDM, MH, and TSBc. Bacteria grown in CDM did not induce macrophage production of TNF $\alpha$  or IL-12 (Figure 22A). In contrast, those grown in MH broth elicited high levels of cytokine (Figure 22A), as has been observed previously [108]. Macrophages infected with LVS grown in TSBc also failed to elicit high levels of both cytokines, similar to what was seen for the CDM (Figure 22A). This was unexpected because TSBc is a complex medium like MH. These data provide convincing evidence that culture conditions used for propagation of *Francisella* prior to performing experiments can significantly

alter the findings of a given study and help explain the seemingly conflicting results previously reported regarding bacterium's ability to activate innate immune responses.

We had previously associated the phenotype of minimal cytokine induction observed following growth of LVS in CDM with an increase in levels of the *Francisella* virulence factor, IglC [108]. We next sought to compare the levels of IglC protein production following growth in the three media examined above. Bacteria grown in TSBc and CDM both expressed higher levels of this protein than did those grown in MH (Figure 22B). Compared to bacteria from MH, those grown in CDM or TSBc contained IglC levels of 2.8 and 7.9 fold, respectively (Figure 22C). IglC levels are higher following growth in CDM (compared to MH), a condition which induces a bacterial phenotype that inhibits macrophage responses (Figure 22A and [108]). Previously, this observation led to the hypothesis that IglC and co-regulated proteins were responsible for the reduced macrophage response to these bacteria. Here, we have shown that bacteria grown in TSBc also exhibit high levels of IglC; while also failing to stimulate macrophage cytokine responses. Taken together, these data show support the previous finding that there is a correlation between IglC expression and the ability of the bacteria to inhibit macrophage cytokine production [108].



**Figure 22: Culture media has dramatic effects on *Francisella* phenotypes.**

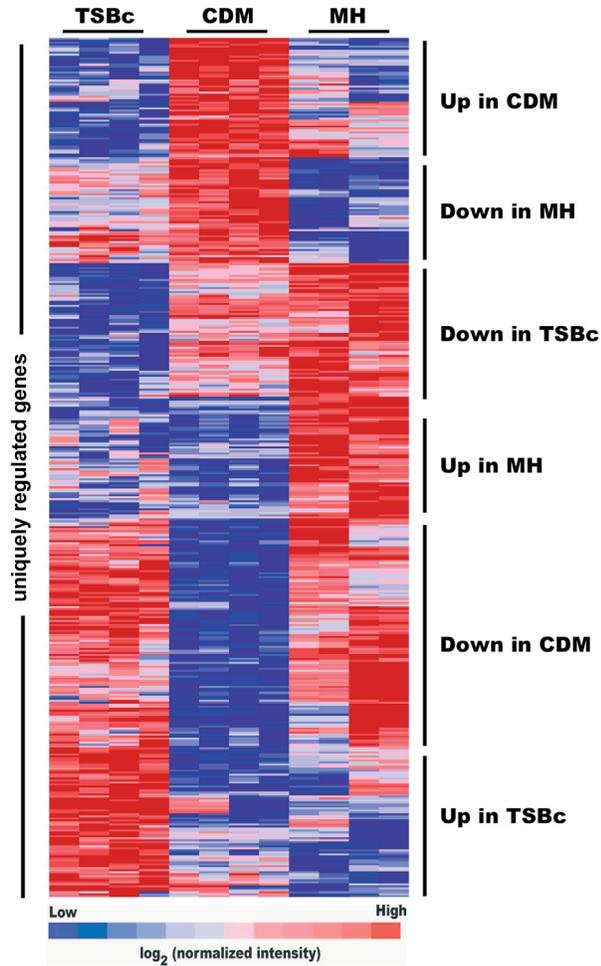
(A) Cytokine production by human monocyte derived macrophages following exposure to *F. tularensis* LVS grown in indicated media at an MOI of 10 for 24 hours. Control wells contained macrophages with no bacteria added (media). Other wells contained macrophages exposed to LVS grown in various culture media as indicated: MH, Mueller Hinton broth; CDM, chemically defined medium, TSBc, tryptic soy broth with cysteine.

(B) Western blots for the *Francisella* virulence factor, IgIC, and loading control Hsp70. Bacteria were grown in the indicated media. Bacterial pellets were lysed and whole cell lysates were loaded into the gel. Samples were normalized by bacterial number (approximately  $1.2 \times 10^6$  bacteria per lane).

(C) Quantitative comparison of LVS IgIC levels following growth in various culture media. CDM and TSBc induced higher protein levels of IgIC than MH broth. Data are presented as fold change in IgIC to Hsp70 ratio CDM or TSBc vs MH (i.e. (IgIC in CDM/Hsp70 in CDM)/( IgIC in MH/Hsp70 in MH)).

#### 4.4.2 Global gene expression changes induced in different growth media:

The clear differences in macrophage activation and virulence factor expression between LVS grown under different conditions suggested that these media have a substantial effect on the bacterium. To fully characterize the potential differences induced by these growth media, we performed a microarray analysis examining gene expression of LVS following overnight growth in each media type. RNA was isolated from LVS was grown in CDM, MH, or TSBc and used to produce labeled cDNA target that was hybridized to a custom *Francisella* microarray. Significant differences in gene expression between LVS under the different conditions were identified using ANOVA with error modeling to reduce false positives [204]. Using ANOVA allowed for the identification of significant gene expression across the three conditions tested. Over 400 genes were identified as being up- or down-regulated following growth in one of the media types. These were then input into Gene Pattern [185] for hierarchical clustering (Figure 23). The clustered data demonstrate clear patterns of expression following growth in each media type allowing the data to be grouped based on expression under a specific condition (i.e. up-regulated in CDM) (Figure 23). Expression levels in LVS growth in CDM, MH, or TSBc increased for 60, 61, and 22 genes respectively (Table 7) and decreased for 168, 53, and 67 genes (Table 8). These data suggest that a substantial portion of the *Francisella* transcriptome is altered based solely on the culture conditions utilized for its growth.



**Figure 23: Unique *Francisella* gene expression changes induced by culture conditions.**

Hierarchical clustering of statistically significant genes, identified using ANOVA, across two independent microarray experiments. Fluorescence intensities across each array were normalized by dividing by the median intensity of that array followed by  $\log_2$  transformation. These values from each experiment were input into Gene Pattern and clustered using the Pearson correlation. Genes clustered into groups representing those up- and down-regulated following growth in each media type. MH, Mueller Hinton broth; CDM, chemically defined medium, TSBc, tryptic soy broth with cysteine.

**Table 7: Genes uniquely up-regulated in each media type.**

CDM		MH		TSBc
FTL_0024	FTL_1099	FTL_0007	FTL_0916	FTL_0012
FTL_0068	FTL_1142	FTL_0087	FTL_0941	FTL_0156
FTL_0080	FTL_1164	FTL_0127	FTL_0999	FTL_0406
FTL_0152	FTL_1171	FTL_0130	FTL_1016	FTL_0492
FTL_0178	FTL_1181	FTL_0131	FTL_1026	FTL_0534
FTL_0181	FTL_1274	FTL_0209	FTL_1060	FTL_0609
FTL_0182	FTL_1382	FTL_0280	FTL_1120	FTL_0690
FTL_0227	FTL_1482	FTL_0286	FTL_1143	FTL_0787
FTL_0235	FTL_1530	FTL_0293	FTL_1157	FTL_0865
FTL_0317	FTL_1531	FTL_0307	FTL_1243	FTL_1074
FTL_0334	FTL_1549	FTL_0326	FTL_1266	FTL_1374
FTL_0354	FTL_1580	FTL_0438	FTL_1309	FTL_1392
FTL_0377	FTL_1609	FTL_0449	FTL_1310	FTL_1489
FTL_0391	FTL_1616	FTL_0523	FTL_1357	FTL_1597
FTL_0397	FTL_1656	FTL_0572	FTL_1399	FTL_1678
FTL_0407	FTL_1657	FTL_0628	FTL_1420	FTL_1805
FTL_0515	FTL_1681	FTL_0639	FTL_1450	FTL_1825
FTL_0544	FTL_1737	FTL_0643	FTL_1451	FTL_1874
FTL_0589	FTL_1794	FTL_0671	FTL_1519	FTL_1961
FTL_0592	FTL_1796	FTL_0672	FTL_1546	FTL_R0025
FTL_0600	FTL_1797	FTL_0673	FTL_1554	FTL_R0032
FTL_0611	FTL_1798	FTL_0674	FTL_1717	FTL_R0038
FTL_0662	FTL_1803	FTL_0675	FTL_1745	
FTL_0693	FTL_1816	FTL_0684	FTL_1788	
FTL_0770	FTL_1823	FTL_0755	FTL_1791	
FTL_0930	FTL_1905	FTL_0813	FTL_1912	
FTL_0951	FTL_1964	FTL_0831	FTL_1913	
FTL_0957	FTL_1965	FTL_0832	FTL_1933	
FTL_0974	FTL_1966	FTL_0881	FTL_1939	
FTL_1045	FTL_1967	FTL_0901	FTL_1960	
		FTL_0915		

**Table 8: Genes uniquely down-regulated in each media type.**

CDM			MH	TSBc	
FTL_0014	FTL_0743	FTL_1279	FTL_0143	FTL_0008	FTL_1478
FTL_0017	FTL_0764	FTL_1302	FTL_0180	FTL_0009	FTL_1511
FTL_0025	FTL_0765	FTL_1303	FTL_0192	FTL_0028	FTL_1540
FTL_0027	FTL_0767	FTL_1334	FTL_0228	FTL_0234	FTL_1552
FTL_0031	FTL_0801	FTL_1338	FTL_0263	FTL_0241	FTL_1579
FTL_0039	FTL_0804	FTL_1341	FTL_0284	FTL_0244	FTL_1738
FTL_0067	FTL_0806	FTL_1360	FTL_0308	FTL_0245	FTL_1773
FTL_0129	FTL_0808	FTL_1363	FTL_0322	FTL_0247	FTL_1799
FTL_0136	FTL_0812	FTL_1461	FTL_0376	FTL_0254	FTL_1910
FTL_0157	FTL_0816	FTL_1477	FTL_0412	FTL_0361	FTL_1931
FTL_0160	FTL_0825	FTL_1485	FTL_0462	FTL_0364	FTL_1935
FTL_0174	FTL_0836	FTL_1488	FTL_0524	FTL_0401	
FTL_0200	FTL_0837	FTL_1492	FTL_0535	FTL_0420	
FTL_0205	FTL_0844	FTL_1494	FTL_0577	FTL_0450	
FTL_0207	FTL_0856	FTL_1498	FTL_0599	FTL_0457	
FTL_0264	FTL_0859	FTL_1499	FTL_0605	FTL_0487	
FTL_0265	FTL_0860	FTL_1500	FTL_0752	FTL_0518	
FTL_0269	FTL_0863	FTL_1522	FTL_0799	FTL_0520	
FTL_0295	FTL_0867	FTL_1545	FTL_0827	FTL_0536	
FTL_0304	FTL_0879	FTL_1556	FTL_0917	FTL_0580	
FTL_0305	FTL_0890	FTL_1594	FTL_0921	FTL_0661	
FTL_0316	FTL_0895	FTL_1614	FTL_0992	FTL_0731	
FTL_0325	FTL_0923	FTL_1707	FTL_1009	FTL_0824	
FTL_0347	FTL_0924	FTL_1729	FTL_1107	FTL_0841	
FTL_0348	FTL_0940	FTL_1742	FTL_1125	FTL_0852	
FTL_0349	FTL_0949	FTL_1743	FTL_1161	FTL_0893	
FTL_0358	FTL_0953	FTL_1763	FTL_1168	FTL_0903	
FTL_0360	FTL_0956	FTL_1787	FTL_1169	FTL_0904	
FTL_0380	FTL_0988	FTL_1789	FTL_1170	FTL_0905	
FTL_0382	FTL_1012	FTL_1800	FTL_1228	FTL_0914	
FTL_0385	FTL_1015	FTL_1808	FTL_1231	FTL_0928	
FTL_0405	FTL_1039	FTL_1812	FTL_1233	FTL_0929	
FTL_0423	FTL_1040	FTL_1832	FTL_1262	FTL_0938	
FTL_0424	FTL_1055	FTL_1834	FTL_1273	FTL_0960	
FTL_0445	FTL_1088	FTL_1866	FTL_1275	FTL_0987	
FTL_0452	FTL_1090	FTL_1868	FTL_1307	FTL_1022	
FTL_0454	FTL_1095	FTL_1879	FTL_1481	FTL_1025	
FTL_0473	FTL_1101	FTL_1894	FTL_1483	FTL_1037	
FTL_0490	FTL_1102	FTL_1895	FTL_1548	FTL_1041	
FTL_0491	FTL_1106	FTL_1902	FTL_1585	FTL_1047	
FTL_0499	FTL_1119	FTL_1923	FTL_1628	FTL_1059	
FTL_0533	FTL_1135	FTL_1936	FTL_1660	FTL_1139	
FTL_0543	FTL_1146	FTL_1938	FTL_1673	FTL_1149	

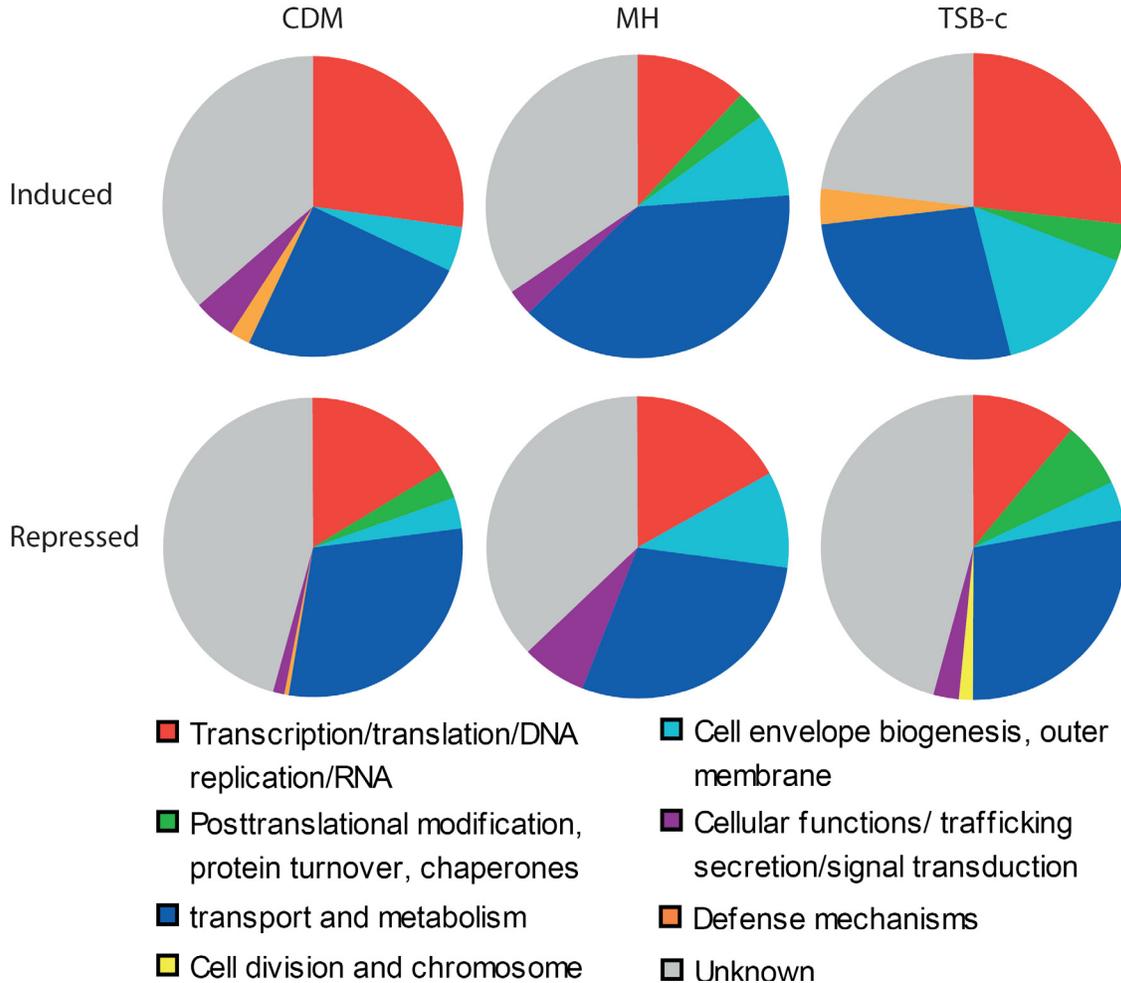
FTL_0548	FTL_1160	FTL_1957	FTL_1700	FTL_1166
FTL_0569	FTL_1202	FTL_R0008	FTL_1722	FTL_1177
FTL_0570	FTL_1204	FTL_R0009	FTL_1762	FTL_1184
FTL_0584	FTL_1216	FTL_R0011	FTL_1782	FTL_1217
FTL_0585	FTL_1219	FTL_R0013	FTL_1784	FTL_1224
FTL_0587	FTL_1221	FTL_R0014	FTL_1817	FTL_1236
FTL_0588	FTL_1223	FTL_R0021	FTL_1862	FTL_1246
FTL_0606	FTL_1225	FTL_R0022	FTL_1863	FTL_1331
FTL_0627	FTL_1227	FTL_R0029	FTL_1915	FTL_1384
FTL_0632	FTL_1245	FTL_R0031	FTL_1944	FTL_1395
FTL_0652	FTL_1248	FTL_R0035		FTL_1404
FTL_0685	FTL_1249	FTL_R0040		FTL_1405
FTL_0700	FTL_1253	FTL_R0050		FTL_1459

In order to more thoroughly examine these results, the uniquely regulated genes were grouped based on the annotated COGs (Clusters of Orthologous Groups). Related COGs were grouped together into the categories shown in Figure 24. The pie charts show the categorical designation of the unique genes that are induced or repressed in each of the three conditions (Figure 24). Each pie represents 100% of the genes and the slices represent the percentage in each category. Many interesting observations can be made from these data.

A large percentage of genes in each pie representing the transport and metabolism category (dark blue). In CDM, there was a significant increase in genes involved in transport/metabolism (dark blue), with 25% of the upregulated genes falling into this category. However, the presence of large slices of this category for each condition tested indicates that the bacteria are undergoing significant changes in their metabolism and transport proteins during growth in these three media types. For example, pathways associated with either oxidation or reduction are differentially regulated. LVS grown in CDM up-regulate genes involved in oxidative phosphorylation (FTL\_1794, FTL\_1796, FTL\_1797, FTL\_1798, FTL\_1816, FTL\_1818, and FTL\_1823). In contrast, bacteria in MH increase expression of pantothenate and CoA biosynthesis (FTL\_0671, FTL\_0672, FTL\_0673, FTL\_0674, and FTL\_0675). Changes in

metabolism may be important for understanding how a bacterium adapts to its environment. For example, in *Mycobacterium tuberculosis*, an alternative metabolic pathway is essential for intracellular growth and virulence [205]. Since growth in macrophages induces a phenotype similar to growth in CDM and TSBc (i.e. minimal cytokine induction during macrophage infection) [132], it is possible that these media more closely mimic the macrophage environment. Evaluating the metabolic pathways induced in *Francisella* by CDM may provide clues about the specific metabolism the bacterium during intracellular growth.

At least 23% of the genes induced in each media type lack a known function. A large portion of the *Francisella* genome consists of hypothetical proteins, the function of which is currently unknown [33]. Many of the hypothetical protein encoded by the *Francisella* genomes are conserved across bacterial species; however there are genes which are specific to members of the *Francisella* genus. Further evaluation of these genes, specifically those up-regulated in condition that lead to macrophage inhibition, could lead to the identification of novel *Francisella* virulence factors.



**Figure 24: Categorical analysis of uniquely induced genes.**

Pie charts represent the COG classification for the genes uniquely regulated in the conditions listed. Each pie represents 100% of the genes exhibiting significant gene expression changes under a given conditions. COG categories were identified for each uniquely expressed gene based on genomic annotation (accession, NC\_07880). The COG categories were grouped into general categories as follows: Categories A, J, K, L, and RNA genes were merged into “Transcription / translation / DNA replication / RNA”; C, E, F, G, H, I, P, and Q were combined into “Transport and metabolism”; COGs N, T, and U were combined into “Cellular functions / trafficking / secretion / signal transduction”; and categories R and S were grouped with uncategorized genes and classified as “Unknown.” Other COG categories listed are reported as they were originally defined. MH, Mueller Hinton broth; CDM, chemically defined medium, TSBc, tryptic soy broth with cysteine.

In order to identify factors that may specifically affect the macrophage response, we first examined the gene expression that correlated with the conditions in which the bacteria failed to activate macrophage responses (Figure 22A). We noted that components of the *Francisella* Type IV pilus were more highly expressed in CDM and TSBc compared to MH broth (up in CDM: FTL\_0390, FTL\_0391; down in MH: FTL\_0799, FTL\_0827). Due to the stringency of the statistical test used for the microarray analysis (see methods), it is not surprising that the entire system was not identified as significantly altered following growth in these media. The type IV pilus genes have homology genes involved in type II secretion [84] and clearly function in this capacity in *F. novicida* [85]. Although type II secretion has been associated with a decrease in virulence of *F. novicida*, these experiments were performed using MH broth and may have missed key effector proteins used by *Francisella* to inhibit macrophage activation [85].

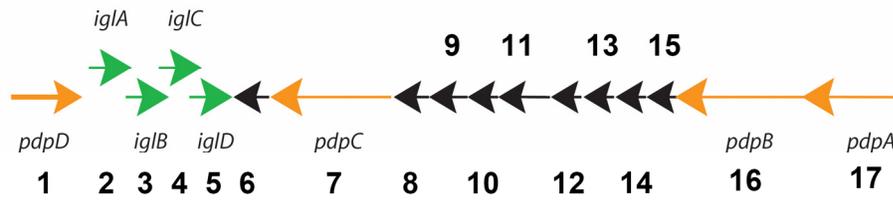
Next, we closely examined the genes found in the only growth condition that lead to high cytokine production from macrophages, MH broth. A total of 53 genes were identified as uniquely down-regulated in MH, (Table 9). By definition, these genes are then expressed at higher levels in the other two media types (CDM and TSBc). One potential mechanism of cytokine modulation is the expression of LPS on the bacterial surface. Wild-type *Francisella* LPS has been shown to be non-stimulatory [88,206] while bacteria with altered LPS structure can activate macrophages [199]. One gene with a potential role in LPS biogenesis is FTL\_0599 a gene that contains a conserved RafG domain which is found in many glycosyltransferases involved in LPS synthesis. This protein could be involved in synthesis of the core oligosaccharide and/or the O-antigen repeating unit [207]. Changes in LPS biosynthesis could

lead to altered LPS structure and subsequent interactions with host cells. Other genes of particular interest are FTL\_0143 and FTL\_1628. Both of these genes encode hypothetical proteins in *Francisella* subspecies, however these proteins do not have orthologs in other bacterial species. *Francisella* specific proteins, such as these, may provide information on the extremely high virulence of these bacteria and a better understanding of their pathogenesis.

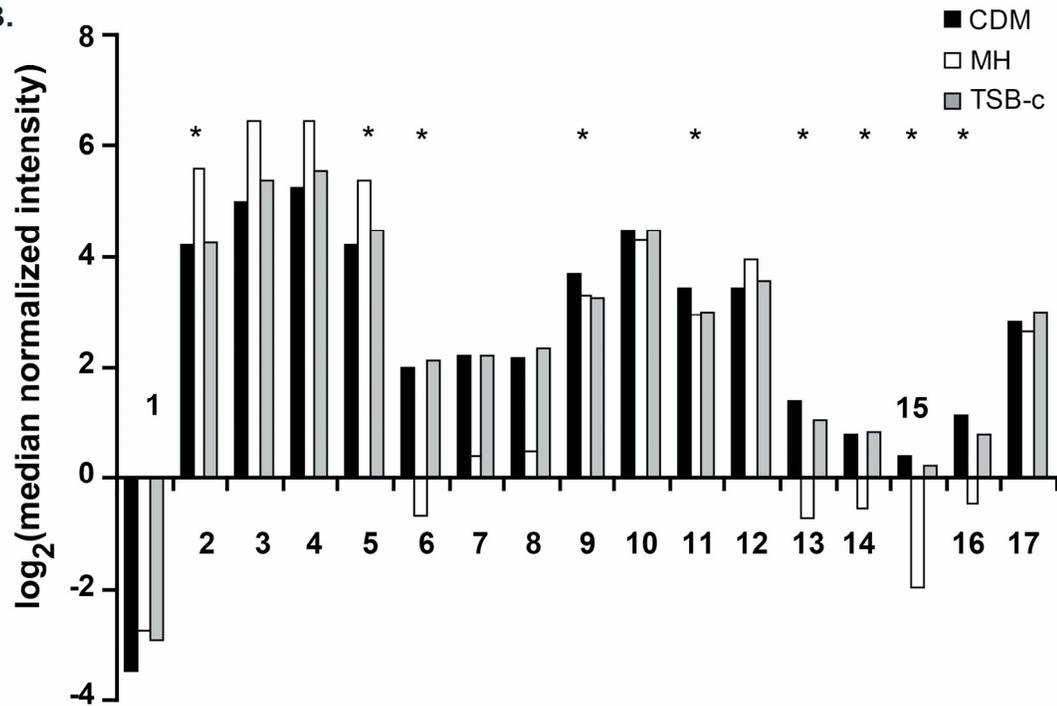
#### 4.4.3 Altered expression of *Francisella* pathogenicity island genes:

To examine the media induced changes in transcription of these known *Francisella* virulence factors, we specifically focused on the genes in the *Francisella* pathogenicity island. Fifty-three percent of the *Francisella* pathogenicity island genes were differentially regulated in depending on the growth media used. Figure 25 shows the log-transformed, median-normalized intensity values for each of the genes in this region of the *Francisella* genome. The genomic arrangement of the pathogenicity island is shown (Figure 25A) and the genes are numbered to correspond with the expression data shown in the bar graph (Figure 25B). Many of the genes were expressed at similar levels in each type of culture media (genes 1, 3, 4, 10, 12, and 17), however significant differences in expression levels were observed for the other genes. The genes that were identified as statistically significant in the original microarray analysis are indicated by asterisks (Figure 25B; genes 2, 5, 6, 9, 11, 13-16).

A.



B.



**Figure 25: Influence of media type on expression of genes within the *Francisella* pathogenicity island.**

(A.) Schematic representation of the LVS pathogenicity island. *Igl* genes are displayed in green, *pdp* genes are displayed as orange, and genes encoding hypothetical protein are displayed as black. Genes are numbered to correspond to part B.

(B.) Gene expression levels for the entire LVS pathogenicity island following growth in each media type. Data are presented as log<sub>2</sub> (median normalized intensity) of values from the microarray experiments. \* = gene identified as having its expression significantly altered in one of the growth conditions.

Together, our data reveal a complex mechanism of transcriptional and translational regulation of the pathogenicity island genes (Figure 22 and 25). Evidence of transcriptional [10,12,71] and post-transcriptional [108] regulation across this locus has been shown previously. Although IglC protein levels are significantly different following growth in the three media types examined (Figure 22), there are no differences in the transcriptional levels of this gene (Figure 25B, gene 4), implying the importance of post-transcriptional regulation. In contrast, other genes in the pathogenicity island exhibit significant differences in mRNA levels, although protein expression has yet to be examined in these conditions (Figure 25B).

The genes in this pathogenicity island are required for intracellular growth of *Francisella*. However, transcription of the entire pathogenicity island can not be associated with the reduced cytokine induction observed for bacteria grown in CDM or TSBc. It is also clear that many signals responsible for regulating these genes. Some genes, including *pdpC* (gene 7), exhibit reduced expression in MH broth, while genes such as *pdpB* (gene 16) are transcribed at higher levels in CDM (Figure 25B). Studies have associated transcription of the entire pathogenicity island genes by a single component of the transcriptional machinery (*mglA*) [10,11,72]. However, our data show many differences in expression of pathogenicity island genes following growth in the three media types. This suggests multiple signals lead to separate transcriptional regulation of various genes in the pathogenicity island. In support of this, low iron induces changes in a subset of the pathogenicity island [12]. These data are supported by a recent report showing individual regulation of the intracellular growth locus genes (*iglABCD*) in *F. novicida* [75].

## 4.5 CONCLUSIONS

Studies of *F. tularensis* have been performed using a variety of types of culture media, including both complex and chemically defined media. Here we have examined the global expression patterns of LVS grown in three commonly used culture media, CDM, MH, and TSBc. These media types induce dramatically altered bacterial phenotypes with differences in cytokine induction and virulence factor expression. Using microarray analysis, we have identified over 400 genes that are uniquely expressed (up- or down-regulated) following growth in one media type. Expression of multiple metabolic pathways, virulence factors, including the *Francisella* pathogenicity island, and other genes were altered in these different media. These media induced alterations may account for contradictory evidence published regarding the ability of LVS to induce cytokine production and wide range of transcriptional changes observed in our studies indicates that care should be taken when designing experiments using *F. tularensis*.

## 5.0 SUMMARY

The work described in this dissertation represents a significant step forward in the attempt to fully understand *Francisella* pathogenesis, specifically the adaptation of this bacterium to the many environmental conditions in which it thrives. It is vital that the bacterium be able to adapt to its various environments, carefully regulating the expression of essential and unnecessary genes. In this way, *Francisella* would alter various aspects of its physiology, including metabolism, nutrient acquisition, secretion, and virulence factor expression, allowing it to succeed in a specific environment.

Growth conditions clearly influence the phenotype of *Francisella*. Chapter 2 shows the effect of growth conditions on the overall phenotype of LVS. Growth of these bacteria to high density in MH broth leads to a bacterial phenotype that activates macrophage cytokine production. Alternatively, sustained growth at low density in MH and any growth in CDM produces an LVS phenotype that does not activate macrophages. In fact, these bacteria actively inhibit macrophage responses elicited through TLR signaling, a common mechanism used by macrophage to detect bacterial products. Along with these phenotypic differences, significant differences in protein expression were induced by these growth conditions. The fact that these two differing culture conditions lead to such significantly different bacterial phenotype indicated a clear bacterial response to some media component.

Further comparison of culture media types also showed significant differences in the activation of macrophages and expression of virulence factors following growth in three media types (MH, CDM, and TSBc). Microarray experiments examining LVS following growth under these conditions led to the identification of over 400 genes that were uniquely regulated in one of three media types examined (Chapter 4). As would be expected for bacteria growing in different environmental conditions, LVS altered the regulation of metabolism and nutrient acquisition systems, adapting for growth in a specific media condition.

The data presented in the three results chapters above contain several interesting observations regarding the genes found in the *Francisella* pathogenicity island. Significant differences in the expression of multiple genes of this locus were identified when comparing bacteria grown in different culture media. The first important observation regards the role of post-transcriptional regulatory mechanisms in the control of these virulence genes. Under multiple conditions it was found that differences in IglC protein levels were not correlated with the gene expression analysis. Comparison of LVS and the isolated ACV variant in Chapter 2 clearly showed differences in IglC levels by immunoblot, though no differences could be demonstrated using Q-PCR. In Chapter 3, different levels of IglC were detected in each media (MH, CDM, and TSBc), though differences in the expression of *iglC* were not observed in the microarray data (Figure 25). Further experimentation is required to determine the exact mechanisms of control being utilized by LVS to regulate protein levels derived from genes like *iglC*.

The basis of many of the experiments presented here are bacterial phenotypes of macrophage activation/inhibition. Early experiments (Chapter 2) indicated a correlation between proteins regulated with IglC and the inhibition of macrophage cytokine responses. LVS

expressed IglC at significantly higher levels than the stimulatory variant ACV. Further evaluation of LVS following growth in the three media types tested in Chapter 4 supported this correlation. LVS grown in CDM or TSBc were non-stimulatory following macrophage infection and expressed increased levels of IglC. The bacteria grown in MH elicited higher cytokine responses from macrophages and expressed the lowest IglC levels. Interestingly, LVS grown in the presence of spermine (Chapter 3) elicit low levels of cytokine from macrophages and also exhibit decreased levels of *iglACD* gene expression (Table 6). Also, the presence of supplemental spermine had no effect on IglC protein levels, with nearly identical bands being observed on immunoblot (data not shown). Taken together, these data show that the inhibition of macrophage responses by *F. tularensis* LVS occurs independently of IglC and the specific *Francisella* protein(s) responsible for this phenotype remain to be elucidated. These data also indicate that the expression of IglC is not regulated by the spermine response, but involves another signal present in both the CDM and TSBc.

The data in Chapter 3 also show that the *Francisella* pathogenicity island is not controlled by a single transcriptional regulatory mechanism. Previous studies have shown that the expression of genes in this portion of the genome are controlled by a single transcriptional regulator, MglA [10,71]. Although these genes were previously thought to be coregulated [10], Figure 25 shows the transcription levels are different following growth in various culture media. Also, both Chapter 1 and 3 provide data supporting a role of a post-transcriptional regulation mechanism in control of the intracellular growth locus genes. In each case, obvious differences were observed in IglC levels when bacteria were grown under different conditions, however significant changes in gene expression could not be detected.

The most important finding derived from this dissertation is the identification of a mechanism of *Francisella* adaptation to the intracellular environment. As discussed above, *Francisella* is able to survive and prosper in a variety of environments, including aquatic environments as well as numerous mammalian hosts and arthropod vectors [3]. Previous study has shown that certain environmental cues are vital for *Francisella* adaptation to the host, though none specific to the intracellular environment had been identified. As with other bacteria, *Francisella* responds to iron restrictive conditions. This condition, which would be encountered in a mammalian host, leads to the increased expression of virulence factors [12]. Macrophage-specific signals that alter *Francisella* gene and protein expression have been identified, including oxidative stress, glucose, and other unspecified factors [13,129-131]. The work presented in Chapter 4 demonstrates the identity of another factor, specific to the intracellular environment in which *Francisella* commonly resides, that significantly alters bacterial gene expression and overall phenotype. This factor, the polyamine spermine, induced gene expression changes similar to what is observed following intracellular growth of LVS, indicating that the bacteria are responding to the high levels of this molecule found in the macrophage cytoplasm. Inactivation of the membrane system controlling the response to spermine led to severe attenuation of LVS in both *in vitro* and *in vivo* model of infection, demonstrating the importance of the ability to adapt in response to spermine. These polyamine transport systems are found in a variety of bacteria and it is possible that other intracellular bacteria are also capable of adaptation in response to spermine. Targeting this system with small molecules or in a vaccine could provide a highly effective treatment against *Francisella* and other pathogenic bacteria.

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Experiments are currently underway to demonstrate that the phenotypes we have observed for *potF-1* are not due to polar effects, but rather due to the specific loss of a transport/sensing mechanism. To this end, we are working on *in trans* complementation of this mutant using a wild-type copy of the gene carried on a *Francisella* shuttle vector. Also, disruption mutants will be created in the other components of the polyamine transport system (*potGHI*) to confirm the role of this system in the spermine response. These mutants will further confirm that the phenotypes observed for the *potF-1* mutant are not due to polar effects as these genes are located in separate genetic loci. Further studies will be performed to fully characterize the *potF* mutant and better understand the role of the spermine response in *Francisella* pathogenesis. These experiments will be expanded upon following the creation of a clean deletion mutant of the *potF* locus. This stable mutant will allow for experiments examining the virulence of this mutant in a murine model of infection. We will also be able to examine the role of cytokine induction/activation on disease pathology and the induction of protective immunity by comparing mice infected with LVS or the stable *potF* knockout mutant.

Together, the data presented here show that *Francisella* responds to different environmental conditions by altering the transcription of a significant number of the genes in its genome. Simple changes in culture media cause altered expression of a significant portion of the LVS genome. A specific signal, spermine, was identified as an important signal that induces gene expression changes in LVS similar to what is observed during intracellular growth. The

ability to sense and respond to this signal is vital to the virulence of *F. tularensis* LVS at the macrophage and whole organism level, implying that the genes regulated by this signal include putative virulence factors, the identity of which remains to be elucidated.

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